Molecular dissection of prefrontal cortex development in health and disease

How serotonin makes up our mind

Lidiane Pereira Garcia

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Doctoral thesis

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by

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For all whom I love, especially Henk

Contents

1

General Introduction

1.1 Central nervous system development

The development of the nervous system during the evolutionary process was fundamental for the emergence of organisms with better adaptive capacity of perception and interaction with the environment[1]. The increase of somatosensory, visual and auditory information led to the development of a neocortex containing increasingly efficient neural sensory and motor maps[1,2,3]. Intelligence brought the need for increased energy and a bigger brain. However, an advanced brain required more time to develop and the organism needed prolonged maternal care[4].

The early development of the central nervous system (CNS) in humans consists of two stages: (1) the embryonic period, which comprises the first eight weeks after ovulation, and (2) the fetal period, which extends from the ninth week until birth[3]. Rodents, such as mice and rats, have a shorter gestational period, between 19 and 21 days[6]. For this reason, the term "embryo" is used to define all prenatal stages of rodent development between fertilization and birth, with the embryonic (E) stage of development indicated by gestational age, and with fertilization counted as E0, approximately 0.5 day after mating[7]. Compared with human staging, the first two-trimesters of gestational development extend from embryonic day (E) 0 to E14.5 in mice and E16.5 in rats, whereas the last fetal trimester ranges from E15-17 to postnatal day (P) 10 in mice[6,7].

The CNS arises with the formation of the neural tube during the third week of gestation, between E20-28 in humans[8] and E8 in mice[1]. The neural tube is the structure that gives rise to the brain and spinal cord[1,9]. The neural tube grows, contorts and transforms into a structure composed of three dilatations (known as primary brain vesicles: prosencephalon, mesencephalon and rhombencephalon) and these expansions will give rise to the main anatomical structures of the adult individual[9] (Fig. 1A). The rostral vesicle is called the forebrain and gives rise to the telencephalon and diencephalon, and these, in turn, will give rise to the cerebral cortex, thalamus, hypothalamus and neurohypophysis[10]. The middle vesicle, the midbrain, will give rise to the cerebral peduncles and the quadrigeminal blade. Finally, the hindbrain will give rise to the metencephalon and myelencephalon, which in turn will form the cerebellum, pons, and medulla oblongata[11] (Fig. 1B). The development of the brain continues after the

postnatal period with an increase in size and also connectivity, and these changes in functional organization are reflected in behavior[12,13,14,15].

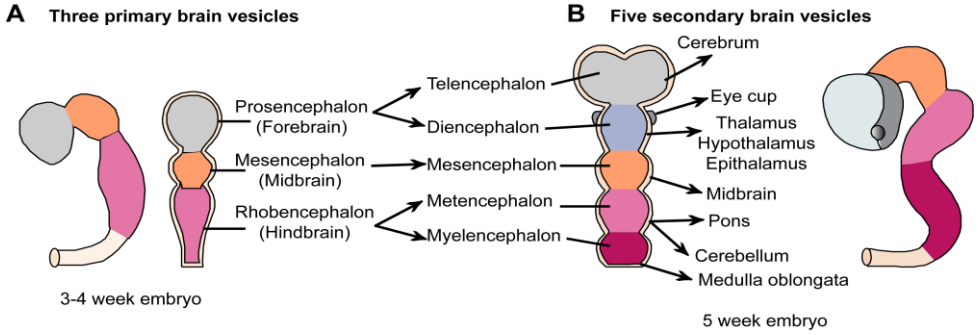

Fig. 1. Embryonic brain development. (A) Following neural tube closure, the embryo begins to expand anteriorly, and the primary vesicles appear in human around E28. These include the Prosencephalon, Mesencephalon, and Rhombencephalon. **(B)** Around E49 secondary vesicles start to emerge. The Prosencephalon is differentiated into the Telencephalon and the Diencephalon, and the Rhombencephalon into the Metencephalon and Myelencephalon. Adapted from Biga et al.[214].

The cerebral cortex is derived from the embryonic telencephalon and consists of a layer of gray matter, which forms a complex pattern of grooves and turns. This is to maximize the surface area of the cerebral cortex, of which 70% is hidden in the furrow depth[16,17]. The cerebral cortex is largely responsible for conscious information, thought, memory, and intellect[19]. It is also related to the organization of movement and to the strategic orientation of complex behaviors over time[20]. The growth of the neocortex during evolution can be characterized as a true phylogenetic explosion, because its neural architecture is different and probably more sophisticated or organized to accommodate more elaborate cognitive functions, which are higher in humans than in any other animal species[18,19].

1.1.1 Corticogenesis

The laminar organization of the neocortex is formed during embryonic development[21]. The neurons destined for different layers of the cerebral cortex are generated from progenitors located in the ventricular (VZ) and subventricular (SVZ) zones of the dorsal telencephalon, being excitatory[21], or from progenitors located in the ganglionic eminence (GE) of ventral

telencephalon which are inhibitory neurons[22]. The neural progenitors can divide to form two other progenitor cells (symmetric cell division), or a progenitor cell and a neuron (asymmetric cell division)[25,26,27]. The shift from symmetric to asymmetric cell division is gradual until the end of neurogenesis[21,23,24], and controlled by intrinsic and extrinsic cell signaling pathways[28,29,30]. In contrast, the glial progenitor cells proliferate in the SVZ of the forebrain and migrate into the white matter and cortex, striatum and hippocampus, where they differentiate into oligodendrocytes and astrocytes[31].

Distinctly organized migratory movements are responsible for the proper positioning of the neurons in the correct cortical layer. There are two types of migration in the cortex: radial and tangential[31,32,33]. In radial migration, the neurons produced in the cortical VZ and SVZ move radially from the progenitor zone to the surface of the brain with a scaffold of radial glial cells[32,33]. The radial glial cells are a special and transient progenitor cell population that can support the migration of many neurons as well as give rise to neurons[34]. In contrast, cortical inhibitory interneurons cross long distances using a tangential migration mode[33]. This route of migration crosses the contour of the development of the cortical mantle tangentially and originates in the GE[35,36]. Tangential migration involves a variety of signaling pathways not seen in radial migration, but both migratory movements are also guided by Cajal-Retzius (CR) cells, situated in the outer marginal zone (MZ), through reelin glycoprotein signaling[36,37].

Neurons use a series of guidance molecules produced locally along their migratory route to direct their movements to the cortex, resulting in an organized six-layered structure (Fig. 2)[38]. The neuronal cortical stratification occurs via the inside-out principle and the first layer to emerge is the deepest layer VI[39]. In mice, the generation of deep cortical neurons of layer VI has its peak around E12, whereas those of layer V have their peak at E13. Neurons of the granular layer IV arise mainly on E14 and the superficial cortical neurons of layers II and III arise around E15-16[21,40]. Around P4 in rodents, all six cortical layers have been formed[40].

The first layer, known as the molecular layer, consists of apical dendrites of pyramidal cells from other layers and CR cells, which are largely derived from the pallium and septum[41]. The superficial layers II/III contain small dense clusters of pyramidal neurons and receive afferents from other cortical layers[21]. Layer IV tends to be thick in the primary sensory cortex and becomes thin in the motor cortex and prefrontal cortex (PFC)[42], being a granular layer rich in interneurons that extend their projections locally to the

cortex and receive thalamic afferents[43]. Layer V contains a large number of pyramidal neurons and is the main source of subcortical efferent fibers and especially well developed in the motor cortex[43,44,45]. The axons of pyramidal neurons exit the cortex and descend through the internal capsule to targets in the brainstem and spinal cord and extend to the contralateral hemisphere[44]. Finally, layer VI is considered the most morphologically diverse as it receives thalamic and cortical afferents[46] (Fig. 2 and 3).

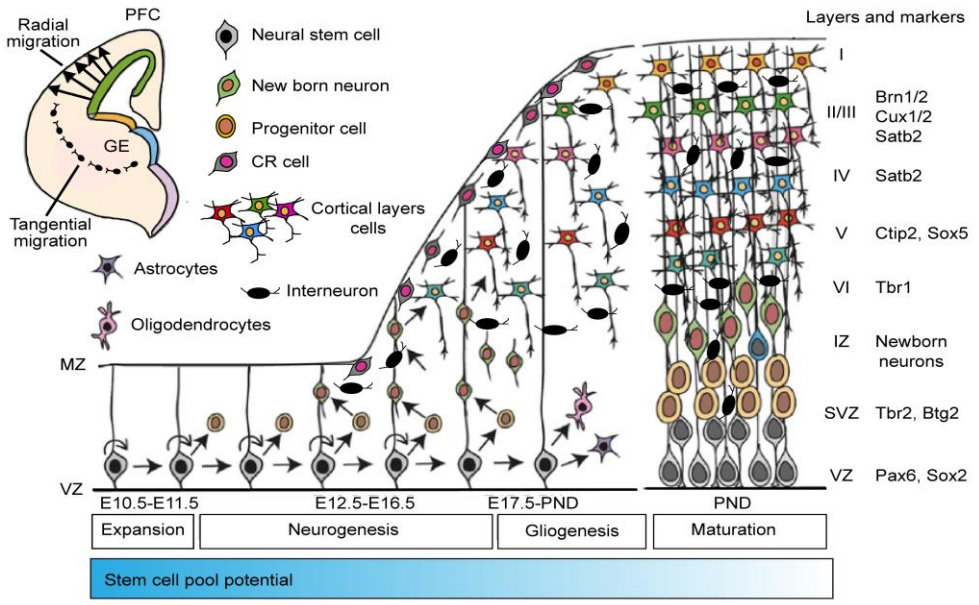

Fig. 2. Stages of corticogenesis. Progenitors generate neurons destined for different cortical layers over time. The newborn cells migrate radially and tangentially guided by soluble factors, such as reelin, to the appropriate cortical position where they undergo differentiation and maturation forming a complex stratified neural network and express different combinations of transcriptional factors. Adapted from Mukhtar and Taylor[215].

Cortical specification is determined by a combinatorial code of gene expression profiles that will result in distinct cortical regions, such as frontal, motor, sensory or visual[21]. Molecular mechanisms, each involving a combination of proteins at varying concentrations, control the regional differentiation of neuronal populations[40,47]. For example, in the proliferative zones a combination of the transcription factors Emx2, Pax6, Coup-TF1 and

SP8 is dynamically expressed[49,51,52,53]. In the normal mouse brain, Emx2 is expressed at high concentrations in the caudal-medial areas, while Pax6 is highly expressed in rostral-lateral areas[49,50] (Fig. 4A). The combination of low levels of Emx2 and high levels of Pax6 induces the motor cortex, while the opposite combination of concentrations induces the visual cortex, and equal levels of both proteins induce the somatosensory cortex[49,50]. In Emx2$^{-/-}$ mutant mice, the Pax6 concentration gradient extends more dorsally. Due to this, the visual areas shrink and the motor areas expand[51] (Fig. 4B). The opposite effect is found when Pax6 expression is suppressed, in response to this the gradual Emx2 expression extends frontally, the visual areas expand and the areas of the motor cortex shrink[50,51] (Fig. 4C).

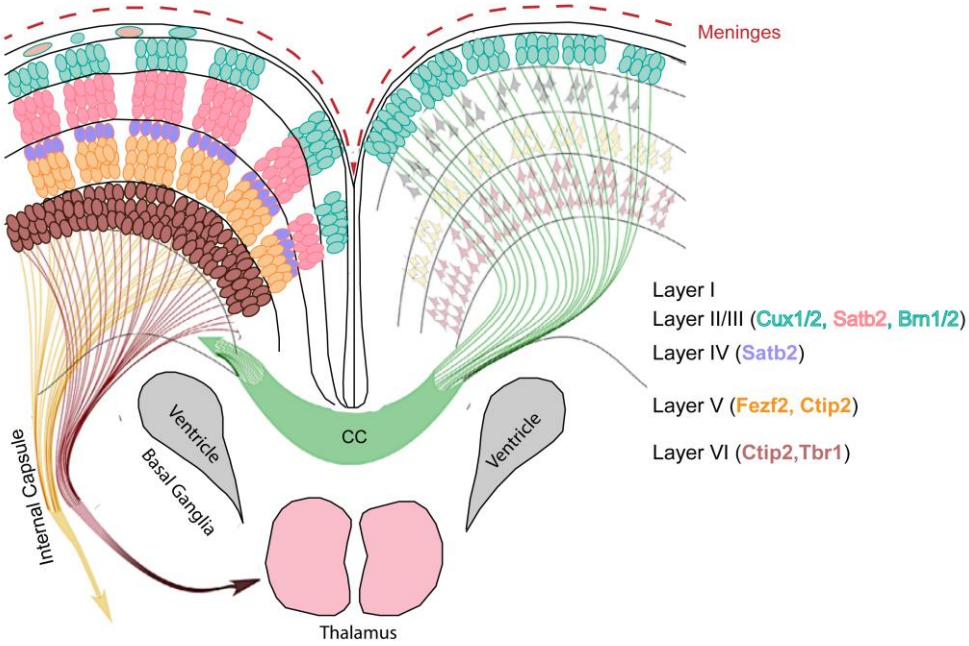

Fig. 3. Prefrontal cortex connections and projections. Layer I: Molecular layer contains few neurons and extensions of apical dendritic tufts of pyramidal neurons and horizontally oriented axons that project locally, as well as glial and CR cells, and receive inputs from thalamus. Layer II: External granular layer contains stellates neurons and small pyramidal neurons that project locally. Layer III: External pyramidal layer with small and medium pyramidal neurons projecting inter-hemispheric corticocortical. Layer IV layer, Internal granular layer, contains different types of stellates and pyramidal cells and receives thalamic projections.

Layer V, Internal pyramidal layer contains large pyramidal neurons and connects with subcortical structures as internal capsule, basal ganglia, thalamus, brain stem and spinal cord. Layer VI layer: Multiform layer contains pyramidal and multiform neurons that project to the thalamus, establishing a very precise reciprocal interconnection between the cortex and the thalamus. Adapted from DeBoer et al.[216].

In cortical layers, dynamic expression of transcription factors is again implicated, in this case in determining the neuronal phenotype. For example, Tbr1, Fezf2 and Ctip2 are involved in the specification of deep layer neurons[45]. Tbr1 specifically regulates the differentiation of layer VI and subplate, and its absence induces cell loss in this layer[55]. Mutant Fezf2$^{-/-}$ and Ctip2$^{-/-}$ animals show a depletion in motor neurons and axon loss in frontal neurons in layer V, respectively[56,45]. On the other hand, the transcription factors Cux1, Cux2, Brn1 and Satb2 are expressed in the initial stages of neuron specification of superficial layers[54,57]. Satb2 confers characteristics to subcerebral, corticothalamic and callosal projection neurons, while the soluble morphogens Fgf8 and Wnt control the relative size and position of these cells[58]. Furthermore, the depletion of Pax6 or Brn1 causes a reduction in neuron generation in the cortical superficial layers[59]. In turn, interneurons can fill all cortical layers depending on chemotactic stimuli, and the final location and specification in parvalbumin, somatostatin and vasoactive intestinal peptide (VIP) interneurons are defined by Dlx1/2, Nkx2.1 and Lhx6[62].

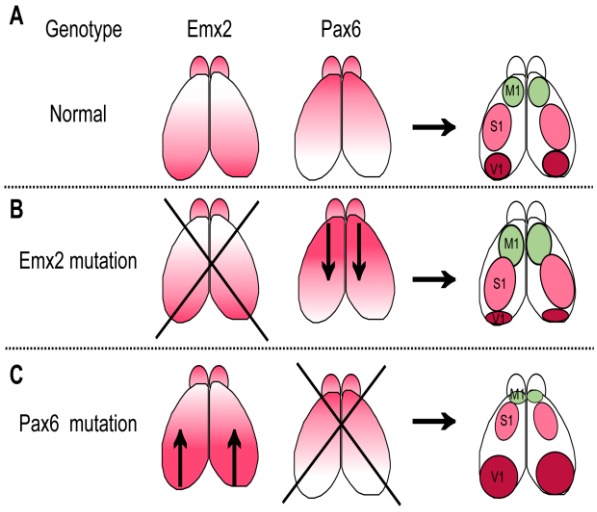

Fig. 4. The effects of different concentrations of Emx2 and Pax6 on the development of cortical areas. **(A)** Normal patterns of frontal and dorsal gradual expression of Emx2 and Pax6. **(B)** Effects of a mutation of Emx2 causing expansion of the sensorimotor areas and shrinkage of the visual area. **(C)** Effects of a Pax6 mutation demonstrating sensorimotor area shrinkage and visual area expansion. Adapted from Bishop et al.[49,50].

The next stages of corticogenesis involve the non-pathological loss of neural elements, through naturally occurring programmed cell death and synaptic pruning, which eliminate transient cell populations such as MZ or SP cells, promote synaptic refinement and correct possible errors in the cortical organization[63,64,65]. Naturally occurring cell death arises in neuronal populations even during the prenatal period and extends to the end of corticogenesis, whereby about 70% of neuronal loss can occur in cortical regions[66]. Pruning of extra connections occurs predominantly postnatally[65]. Both processes are controlled by neurotrophic factors derived from neurons or other brain cell types such as microglia, astrocytes and oligodendrocytes, and represent axonal integrity and neural survival signals[67].

Finally, the last stage of corticogenesis involves neuronal myelination that begins around E19 in mice, when the oligodendrocyte begins to differentiate and increases the expression of myelin extending its processes towards the neuronal axon[68]. The increase in axonal conduction velocity and synaptic refinement is indicative of neuronal maturation[69].

In summary, the dynamic processes of proliferation, glial and neuronal specification and migration, followed by differentiation, cell death, and maturation are largely prenatal (except for synaptic pruning and neuronal myelination) and essential for the correct cortical organization[21,70] (Fig. 2). These cortical events are controlled by intrinsic factors consisting of a complex program of expression of transcription factors and neurotrophic factors, combined with extrinsic factors that involve signaling of afferents from other noncortical regions as well as local guidance.

1.1.2 PFC development

From an evolutionary point of view, the PFC is the most recent brain structure. In human, the PFC comprises 29% of the cortical surface, consists of a large variety of cells, and is conventionally divided into six layers[3,4,6,17]. The relatively large size of the human PFC presumably indicates that the cortex is the substrate for higher-order cognitive functions[6]. In rodents, a major division of the PFC involves the ventromedial area, which can be subdivided into the cingulate, prelimbic and infralimbic cortex[71,72,72]. The PFC receives projections from cortical, sensory, motor and limbic areas[74]. The connections with temporal and parietal areas are associated with cognitive functions, whereas connections with the amygdala and hippocampus are linked to emotions and memory[72]. Most prefrontal areas

have six layers, including a granular IV layer, except for the orbitofrontal and posterior mediastinal areas, and belong to the cortical-limbic system[1].

Magnetic resonance imaging has shown that human PFC development is characterized by growth in early childhood, a decrease in size in adolescence, and then a slight increase and stabilization in adulthood[84], followed by a decline in the volume of gray matter and an increase in white matter volume[81,82]. Cortical gray matter reaches its maximum volume in the frontal lobe between the ages of 11 and 12 years, being lately in the chronological development, when compared to other areas[87,86]. This contrasts with the cortex as a whole, where gray matter increases mainly from infancy to age 6 to 9 years[86]. During this period of brain development, the dorsolateral and medial PFC expands almost twice as much as some other dorsal regions, but mature late in relation to a large part of the remaining cortex of the sensory-motor regions and other[81]. It is believed that this pattern is linked to the maturation of cortical circuits underlying complex frontal lobe functioning, including language, decision making, attention control, and memory[84,85,86].

1.2 Serotonergic and dopaminergic system development

1.2.1 Serotonergic system development and impact on mPFC development

Serotonin (5-hydroxytryptamine or 5-HT) is a monoamine neurotransmitter and neurotrophic factor that affects brain functioning from emotions to motor skills[88]. In animals, 5-HT is synthesized from the amino acid L-tryptophan by a metabolic pathway consisting of the enzymes tryptophan hydroxylase (TH), aromatic amino acid decarboxylase (AADC) and pyridoxal phosphate coenzyme[88,89], and was isolated from serum for the first time in 1948[90].

During brain development, the serotonergic system emerges in the brainstem with a group of cells that will form the raphe nuclei[91], between E9.5-E12 in mice (E10.5-E13 in rats) and from the fifth to the seventh week of gestation in humans[91,92]. This system is tightly controlled by a complex of signaling and gene regulatory networks, such as sonic hedgehog (Shh) signaling and the Nkx2-2/Lmx1b/Pet1 cascade[93]. However, brain synthesis of 5-HT contributes to its PFC levels only around E14. Prior to this developmental time point the PFC receives 5-HT via the placenta or myenteric plexus[94], from E10-E11 until E18, when the cerebral serotonergic

nucleus is developed, and it is able to supply the whole brain[94]. The raphe nuclei are grouped into nine clusters: the caudal division (B1-B4, including pale raphe, obscurus, magnus and pontis), the rostral division (B9), the dorsal division (B6, B7) and the median division (B5, B8), with the last two divisions projecting to the PFC[88,95]. The proportion of serotonergic neurons varies from 50% in the dorsal raphe (DRN) nuclei to 21% in the raphe median (MRN), 19% in rostral (RRN) and less than 10% in the raphe caudal (CRN)[96,97] (Fig. 5A).

Axons from the serotonergic neurons of the raphe nuclei send their projections to the frontal targets and reach the PFC, via the septum and internal capsule, around E14 in mice (E16 in rats), but only around E16 they reach the mPFC[98] (Fig. 5B). In the mPFC, the serotonergic axons are initially divided into two major bundles[98,99]. The first bundle present in the MZ is in close proximity to CR cells, which may control the release of reelin and also guide neuronal migration and organization[100,101,102]. The second bundle is found below the CP, especially in the IZ and SP, where proliferating and migrating cells are located[98,102]. At the end of corticogenesis, the serotonergic axons gradually broadcast by sending numerous branches to the CP[103,104]. Serotonergic axons then progressively distribute uniformly in different cortical subdomains. However, only at PND28 serotonergic neurons show their mature pattern of innervation[105]. Factors regulating serotonergic axon growth and final innervation pattern may involve S-100, a protein secreted by astrocytes, which acts as a guidance molecule for the migration of raphe neurons[103,104,105].

Cortical layers IV and V receive inputs from DRN which are anatomically thin and exhibiting numerous branches with varicosities[102]. In contrast, MRN projections are characterized by large spherical varicosities that can form true chemical synapses[102,106]. They preferentially forage into layer I and the lower white matter, and surround the neuronal cell bodies and proximal dendrites, and preferentially contact interneurons[102,103,106]. In the embryonic and mature mPFC, infralimbic and prelimbic cortex neurons also send their afferent projections to the raphe nuclei, and thereby control serotonergic release in a feedback mechanism, via serotonin autoreceptors[107,108].

Acting on postsynaptic and presynaptic receptors, 5-HT is involved in cognition, mood, impulse control, and motor functions[109]. It modulates the activity of different types of neurons and influences the release of other neurotransmitters such as glutamate, GABA, acetylcholine, and dopamine[103]. In addition, 5-HT seems to play an important role in cortical development by

controlling cell proliferation, migration, organization through signaling via CR cells, apoptosis and neuronal maturation[102,110], thus demonstrating the important neurotrophic role of 5-HT in cortical development in critical events that will last until adulthood.

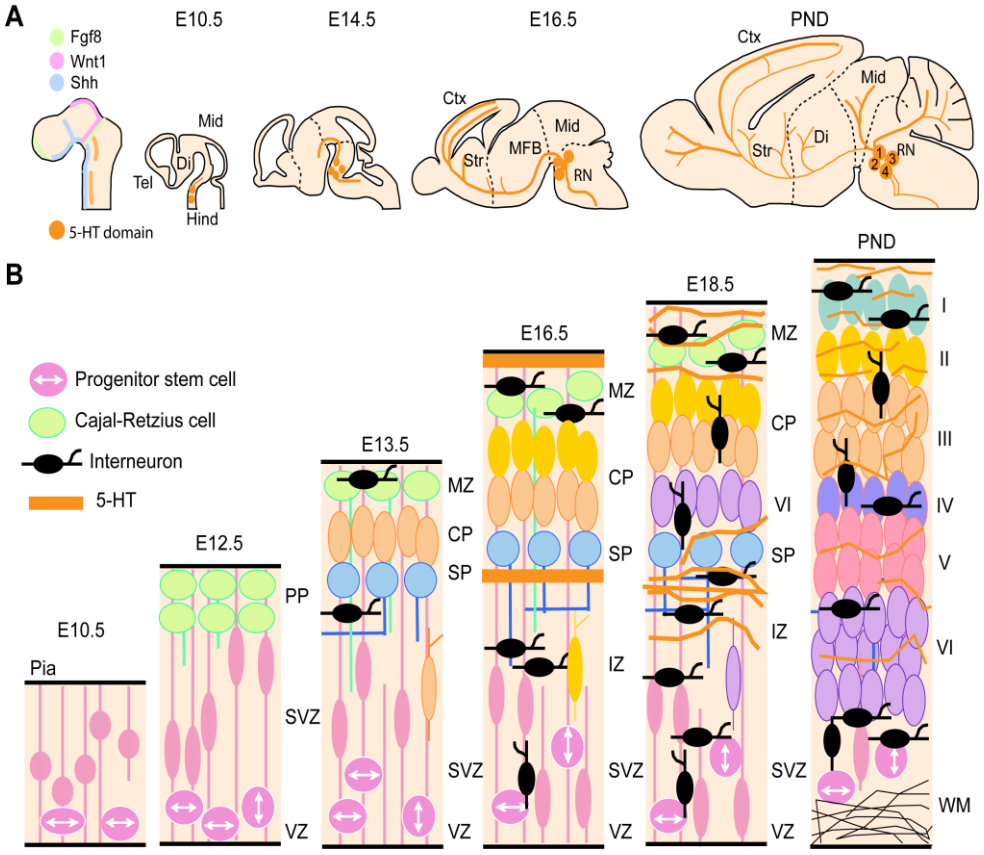

Fig. 5. Development of the serotonergic system and its mPFC projections. **(A)** Sagittal sections of developing brain demonstrating the proliferative onset of cells, in the hindbrain region, that will form the raphe nuclei at E10.5. Its serotonergic afferents will innervate the forebrain. **(B)** Cortical development in relation to 5-HT sources. Corticogenesis begins from embryonic day E10-11 through an intense proliferation of progenitor cells located in the VZ that will give rise to glutamatergic cells. Around E16.5, serotonergic afferences reach the sub-domains of mPFC in two bundles, one in MZ and one under SP, and make connections with CR, interneurons and proliferating cells. With layering and differentiation and cortical maturation, serotonergic innervation is distributed evenly across the layers. Layers V and VI preferably receive afferents from the dorsal raphe (B6, B7), while layer I receives

afferents, mainly from the median raphe (B5, B8). Midbrain (Mid), Hindbrain (Hind), Telencephalon (Tel), Cortex (Ctx), Striatum (Str), Medial forebrain bundle (MFB), Raphe nucleus (RN), Prelate (PP), Subventricular zone (SVZ), Ventricular zone (VZ), Cortical plate (CP), Subplate (SP), Intermediate zone (IZ), White matter (WM). (1) Medial raphe (B5, B8), (2) Raphe rostral (B9), (3) Raphe caudal (B1-B3), (4) Dorsal raphe (B6, B7). Adapted from Vitalis and Parnavelas[102], and Niederkofler et al.[117].

1.2.2 Dopaminergic system development and its relation to the mPFC

DA (or 3,4-dihydroxyphenethylamine) is a neurotransmitter and neurotrophic factor from the family of catecholamines that plays several important roles in the brain and body[111,113]. DA is synthesized from the essential amino acid phenylalanine and from the non-essential amino acid tyrosine[111,112]. DA itself also acts as a precursor in the synthesis of the neurotransmitters noradrenaline and epinephrine[112].

During development, dopaminergic progenitors are generated in the mesencephalon around E10 in mice and E12 in rats[116,117,118]. Expression of TH is the first sign of acquisition of the dopaminergic neuronal phenotype and occurs shortly after the final mitosis of the dopaminergic neural progenitors, starting at E11.5, while they are actively migrating to their final positions[119,120,121,122]. In the adult brain, dopaminergic neurons are a heterogeneous group of cells located in the midbrain, diencephalon and olfactory bulb[113]. The dopaminergic neurons from midbrain send axonal projections to the PFC areas, and these cells are located in the retrorubral field (RRF, A8), compact substancia nigra (cSN, A9) and ventral tegmental area (VTA)[114,115].

Starting at E11.5, dopaminergic neurons extend their axons frontally through the striatum, amygdala, septum and PFC, where the mesolimbic and mesocortical routes originate[114,123], the axons reach the PFC, in E14 in mice, but only E16 in the mPFC[114] (Fig. 6A). Distributed in two main bundles, one in MZ where CR cells are, and the second bundle in CP and SP[124]. Due to its proximity to CR cells, DA also participates in important stages during corticogenesis including neuronal migration, and also plays an important role during neuronal differentiation[125,126] (Fig. 6B).

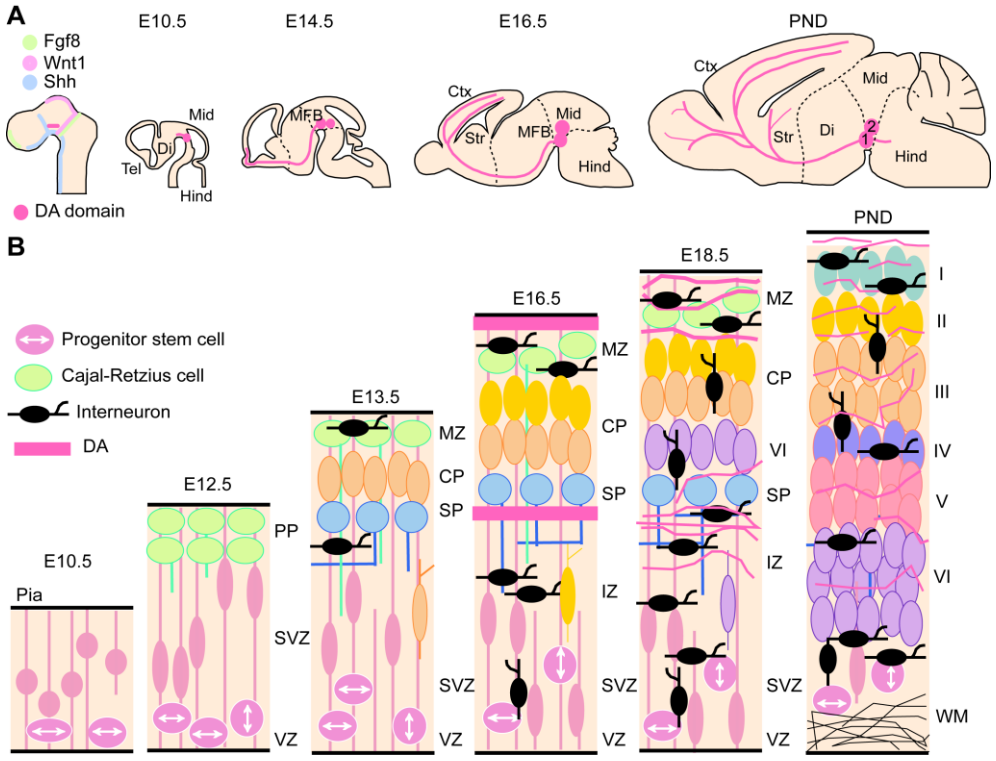

Fig. 6. Development of the dopaminergic system and its projections to the PFC. **(A)** Time point development of the origin of dopaminergic system in sagittal cuts. Cells located in midbrain send their projections to frontal targets around E10.5. These cells travel via the medial forebrain bundle and the striatum and arrive in the PCF around E14. **(B)** Development of mPFC in relation to dopaminergic innervation. Around E16.5, the axonal projections coming mainly from VTA and SN arrive at MZ and SP. It can thus stimulate and organize the neuronal migration via CR in the MZ and induce cell proliferation in proliferative zones. After maturation the dopaminergic system is distributed throughout all cortical layers thus making synapses with different cell types, both excitatory and inhibitory. Subventricular zone (SVZ), Ventricular zone (VZ), Midbrain (Mid), Hindbrain (Hind), Telencephalon (Tel), Cortex (Ctx), Striatum (Str), Medial forebrain bundle plate (MFB), Subplate (SP), Intermediate zone (IZ), White matter (WM). (1) Ventral tegmental area (VTA), (2) Substance nigra (SN). Adapted from Prestoz et al.[114] and Niederkofler et al.[117].

In the early postnatal days, the density and morphology of dopaminergic fibers increases[117]. The excitatory dopaminergic receptors D_1 and D_5 or inhibitory D_2, D_3 and D_4 are overproduced in early adolescence,

followed by pruning that is more evident in the subcortical than pre-frontal regions[127,128]. The net effect of increased dopaminergic projection to the PFC with less pruning is a change in the relative balance between subcortical and cortical dopaminergic systems with dominance of the mesocortical dopaminergic system, especially through pyramidal neurons in memory tasks[127,129]. In summary, DA is responsible for significant changes in cortical development and neural circuits involved in emotional and cognitive regulation, from the embryo to childhood, adolescence and the adult brain.

1.2.3 Serotonin and dopamine interrelationship

During brain development, the serotonergic and dopaminergic systems reside close to each other, are influenced by a set of intercellular signaling factors, receive afferent input from the same brain regions, and send efferent projections to common targets, such as the mPFC, and in a similar time window. These data suggest an interrelationship between these two systems[102,117,130,131]. In addition, there are connections between serotonergic nuclei (DRN and MRN)[132] and dopaminergic nuclei (VTA, SN), specially through GABAergic interneurons[133] (Fig. 7).

The support for such an interaction is provided by experiments in which one of the systems is chemically or genetically damaged pre- or perinatally (Table 1)[117]. 5-HT seems to inhibit and oppose dopaminergic activity, particularly in relation to the role of DA in aggressive and impulsive behaviors[117,137,138]. When dopaminergic neurons are damaged in their supply of VTA and SN, they actually cause lesions in the serotonergic system, both at the molecular/cellular and behavioral levels[134,135]. Chemical ablation of the dopaminergic neurons causes disturbances in the serotonergic innervation of the mPFC[126]. In contrast, it appears that dopaminergic innervation of the mPFC is suppressed by serotonergic signaling, since neonatal chemical damage of serotonergic DRN neurons led to an increase in dopaminergic fibers in the mPFC[136]. The relative imbalance in serotonergic-dopaminergic activity during the final phase of cortical development may explain situations of increased sensitivity, resulting in a higher prevalence of risk behaviors[139]. The interaction between the monoaminergic and catecholaminergic neuronal systems, both during perinatal development and in the adult organism, generates a developmental window of vulnerability that extend to the field of psychiatric illness[130].

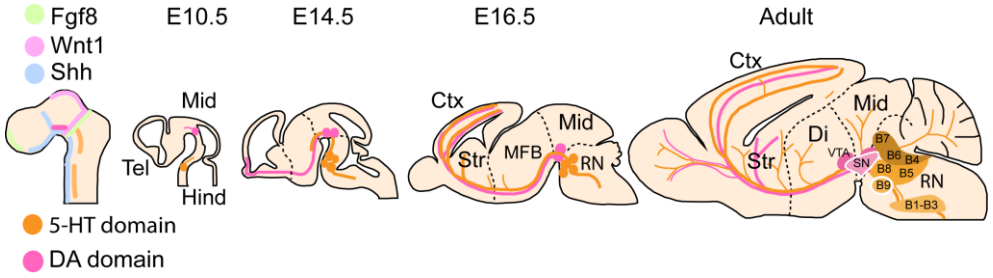

Fig. 7. Interrelationship between DA and 5-HT system development. Drawings in a sagittal view of mouse brain show the anatomically proximity of the source of 5-HT (orange) and DA (magenta) systems, as well as the parallel projection targets in embryonic and adult stage. Midbrain (Mid), Hindbrain (Hind), Telencephalon (Tel), Cortex (Ctx), Striatum (Str), Medial forebrain bundle (MFB), Prelate (PP), Subventricular zone (SVZ), Ventricular zone (VZ), Cortical plate (CP), Subplate (SP), Intermediate zone (IZ), White matter (WM). Ventral tegmental area (VTA), Substancia nigra (SN). Raphe pallidus (B1), Raphe obscurus (B2), Raphe magnus (B3), Dorsal raphe (B7, B6, B5), Median raphe (B8, B5) and Rostral raphe (B9).

Table1

Pharmacological interrelations between Serotonin (5-HT) and Dopamine (DA) in treatment strategies of mental disorders

Drug	Drug target	Drug effect on 5-HT levels or 5-HT neurons	Ref
Dopamine	Nonspecific DA receptor agonist	Increased mPFC 5-HT levels	Petty et al., 1994[217]
Apomorphine	Nonspecific DA receptor agonist, used systemic	Increased DRN 5-HT release; increased 5-HT neutron firing rate.	Aman et al., 2007.[218]Lee EH, 1987.[219]Martin-Ruiz et al., 2001.[220]
	Nonspecific DA receptor agonist, used systemic, local mPFC	Increased mPFC 5-HT levels	Petty et al., 1994. [217]

Drug	Drug target	Drug effect on 5-HT levels or 5-HT neurons	Ref
Haloperidol	D2-like receptor antagonist; used systematic	Decreased in basal DRN 5-HT levels	Martín-Ruiz et al. 2001.[220]
	D2-like receptor antagonist; local mPFC	Increased mPFC 5-HT levels	Petty et al., 1994.[217]
SSRI in combination with methylphenidate	5-HT reuptake inhibitor	Increased in PFC DA release compared to methylphenidate alone; no such effect in the nigrostriatal pathway	Weikop et al., 2007.[221]
DRI	Dopamine reuptake inhibitor	Increased extracellular DA levels; increased 5-HT neuron firing rate	Guiard et al., 2009.[222]
TRI	Triple reuptake inhibitor	Increased extracellular levels of all monoamines; incomplete suppression of 5-HT neuron activity, in contrast to SSRIs alone	Guiard et al., 2009.[222]

mPFC, medial prefrontal cortex ; SNc, substantia nigra pars compacta; DRN, dorsal raphe nucleus. Adapted from Niederkofler et al[117].

1.3 Brain disorders

1.3.1 Brief history of the role of the brain in mental illness

Many cultures have seen brain disorders as a form of religious punishment or demonic possession. In ancient Egyptian, Persian, Greek, and Roman writings, mental illness was categorized as a religious problem[140,142]

Fig. 8. Mental disorders. Watercolor drawing of an anatomy book from 1488 in Persia. Demonstrates beliefs about the correspondences between certain forms of mental illness and specific parts of the body. Source: Tashrih al-Badan, 1488. The National Library of Medicine[142].

(Fig. 8). In the fifth century BC, Hippocrates by using medication and change of environment, was the first to be able to treat mental illness without religious techniques [141,142]. During the Middle Ages, it was believed that the mentally ill were possessed or in need of religion. Negative attitudes toward mental illness persisted until the eighteenth century, leading to the stigmatization of the disease and unhygienic (and often degrading) confinement of mentally ill individuals[142]. In the 1840s, activist Dorothea Dix lobbied for better living conditions for the mentally ill after witnessing the dangerous and unhealthy conditions in which many patients lived[143]. This model of institutional hospitalization, in which many patients lived in hospitals and were treated by professionals, was considered the most effective way of caring for the mentally ill until then[141], also well-received by families and communities who struggled to care for mentally ill relatives[143]. Even providing access to mental health services, state hospitals were often underfunded and accused of poor living conditions and violation of human rights. This led to ambulatorial treatment, especially after the development of antipsychotic drugs, in the mid 1950s[144]. Deinstitutionalization efforts reflected a broad international movement to reform mental health, based on the belief that psychiatric patients would have a better quality of life if treated in their communities rather than in large, undifferentiated and isolated mental hospitals[145]. Although large psychiatric hospitals are still common in some countries, particularly in Central and Eastern Europe, the deinstitutionalization

movement has been widespread, drastically changing the nature of modern psychiatric care[141,144]. But what is a mental disorder?

A mental disorder is a dysfunction in the developmental, psychological or biological processes underlying brain function[145]. Clinically this dysfunction culminates in a syndrome characterized by disordered emotional regulation, cognition or behavior of an individual[147]. There are many different categories of mental disorders involving behavior and personality. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) of the American Psychiatric Association, the list of major disorders includes anxiety disorder such as panic syndrome, generalized anxiety, stress, or phobia; bipolar disorder, which alternates periods of depression and periods of mania; depressive disorders, in which there are various types of depression; obsessive-compulsive disorder (OCD); psychotic disorders, such as schizophrenia or delusional disorder; eating disorders, such as anorexia or bulimia; post-traumatic stress disorder (PTSD); personality disorders, such as paranoid, antisocial, borderline, histrionic or narcissistic types; disorders related to the use of substances, such as illicit drugs, alcohol, drugs or cigarettes; neurocognitive disorders, such as delirium, Alzheimer's or other dementias, and finally neurodevelopmental disorders, such as intellectual disabilities, communication disorders, autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD)[145,146,147].

1.3.2 The roles of 5-HT and DA in brain disorders

In addition to higher order cognitive functions and emotional regulation, the cerebral cortex integrates sensory and motor cortex through a complex neural network[148]. Precisely timed construction disturbances of the PFC, during development, affect the cortical structure and may confer risk for various neuropsychiatric disorders[149,150]. The involvement of 5-HT and DA in critical components of corticogenesis, including proliferation, migration of neurons and glia cells, with differentiation including adequate growth and orientation of axons towards their targets, are genetically determined and conserved phylogenetically, and their levels should be kept normal during such processes[102,103,158]. The dorsolateral cortex and anterior cingulate cortex are two PFC regions that are affected in autism, schizophrenia, depression, and anxiety[151,152,153]. For example, in autistic spectrum disorder (ASD), a low dopaminergic activity of the mPFC has been identified[154,155,156]. The PFC laminar organization of autistic children demonstrated an increase in neuronal density, suggesting that there are more neurons than expected in that

region[162,163]. However, at school age these differences disappear, and the cerebral volumes are normal or even slightly lower than normal[164,165]. It is believed that in schizophrenia there is a subcortical DA excess and a cortical DA deficit[166]. The serotonergic influence involves increased expression of 5-HT_{2A} and 5-HT_{1A} receptors in the PFC, hippocampus and thalamus in schizophrenic patients compared to controls[166,167]. Morphologically, the anterior cingulate cortex is characterized by smaller and more widely spaced neurons in layer II, and reductions in synaptic and dendritic density[168,169].

Biological, genetic and psychosocial factors interact with each other and may cause altered availability of biogenic brain amines, especially 5-HT, noradrenaline and DA, and thereby increase risk for depression and increased anxiety[170]. A polymorphism in the promoter region of the gene encoding the 5-HT transporter (5-HTTLPR) has been associated with depression and high levels of anxiety[171]. Since the serotonin transporter (5-HTT) acts as a regulator of the 5-HT levels in the synaptic cleft, its absence causes an excess of this neurotransmitter[172,173]. 5-HTTLPR is a repeat polymorphism with long (1) and short (s) alleles. The allele (s) is associated with lower 5-HTT expression and function, and increases vulnerability for anxiety and depression in human[171,172,173]. In rats, 5-HTT knockout (5-$HTT^{-/-}$) induces various behavioral phenotypes, which include anxiety, depression-like symptoms, and motor and cognitive delays[174,175-178]. The 5-$HTT^{-/-}$ genotype induces an increase of serotonergic innervation in the mPFC and damages the cortical layering during development[179]. In addition, an increase of extracellular 5-HT via a pharmacological block of 5-HTTs causes an interference with the development of many areas of the brain, such as the barrel cortex, amygdala, hippocampus and hypothalamus[180,181,182,183]. Selective serotonin reuptake inhibitors (SSRIs) are the most common medication prescribed during pregnancy for 20% of women to treat maternal depression, or during postpartum depression, where nearly 40% of women can develop this disturbance[182]. Prenatal SSRI exposure may increase risks for depressive, anxiety and abstinence behaviors[184], delays in motor development[185], abnormal psychomotor development such as hearing, vision and motor attention[186], and autism spectrum disorders[187]. In another aspect, the non-selective serotonin reuptake blocker, such as Efavirenz (EFV), has also been associated with disturbances in the serotonergic system[203,204,205]. Efavirenz is the first line treatment for HIV-positive pregnant women, decreasing the chance of transmission of the HIV virus from mother to fetus to less than 5%[206]. However, EFV interacts with various receptors and transporters, including dopaminergic, serotonergic and noradrenergic[204], and

has in adults been associated with side effects such as increased anxiety and depression[205]. In adult rats exposed to EFV, there was cell loss in various areas of the brain such as hippocampus, striatum, and cerebellum[207-209]. Prenatal exposure to EFV has been associated with deformities in nervous system formation, but with divergent results between studies in humans and animal models[210-213]. Studies demonstrating the perinatally toxicity of perinatal EFV exposure to rats and its what their consequences on cortical and ultimately behavioral development have not yet been performed. In contrast, adult EFV-exposed rats have been examined and showed behavioral changes such as an increase in anxiety, indicating a possible side effect of EFV on the serotonergic system through the increase of 5-HT levels and its risks for cortical development[203,204,205].

In summary, different types of brain disorders have in common changes in the levels of 5-HT and morphology of the serotonergic system. During pre- or perinatal development these cause changes in cellular, structural and neural connections, in addition to altering other systems such as the dopaminergic system. These changes have an impact on adult life and are modulated by environmental factors.

1.4.0 Aim and outline of the thesis

The aim of this thesis is to investigate how changes in 5-HT levels, via genetic or pharmacological manipulation, interfere with the intrinsic and structural mechanisms that control the development of the mPFC. In addition, we seek to identify possible interrelations of the serotonergic system with other systems, such as the catecholaminergic system, and how they together affect the stages of corticogenesis during neurodevelopment. Finally, we examine how these prenatal changes are reflected in postnatal and adult behavior.

In Chapter 2, we use mice to study the pattern of normal brain development in a dorsal-frontal wave at different embryonic and postnatal stages. We investigate the temporal and structural aspects of the prefrontal and somatosensory cortex, and various cortical developmental processes such as proliferation, migration, differentiation, and maturation. In addition, we show how the serotonergic and dopaminergic systems develop in different cortical areas at different developmental time points. The rationale here is to first understand how mPFC subareas normally develop before we enter into the use of different genetically or pharmacologically manipulated animal models in the next chapters.

In Chapter 3, we use a rat mutant for 5-HTT (5-HTT$^{-/-}$) displaying a disturbance in the serotonergic system. We investigate the intimate relationship between the developing serotonergic and dopaminergic systems, e.g. changes in the frontal projections of both systems. We also identify how disturbances in serotonergic and dopaminergic innervations of the mPFC are able to affect the cortical organization, especially through synaptic contacts with CR cells.

The aim of Chapter 4 is to investigate the changes in serotonergic projection and the identity of the mPFC layers when extracellular 5-HT levels are increased due to the absence of the 5-HTT in rats (5-HTT knockout (5-HTT$^{-/-}$) rat). We use transcription factors as layer markers and follow changes in columnar organization. In addition, we demonstrate changes in neuronal maturation and cell survival, via markers such as NeuN and Caspase-3, respectively. Furthermore, we employ the elevated plus maze test in adolescent and adult animals to study the emergence of anxiety in a given time window. As such, we show that in the absence of the 5-HTT structural changes in the mPFC are reflected at the behavioral level.

In Chapters 5 and 6, we examine the short- and long-term behavioral and neurodevelopmental consequences of perinatal exposure of pregnant rats to the HIV drug EFV or vehicle (from day 1 of gestation to P7). In Chapter 5, we focus on male offspring of the EFV- and vehicle-exposed rats and explore the development of the primary motor cortex, use behavioral tests during the first three postnatal weeks, adolescence and adulthood, and conduct brain immunohistochemical analyzes. Perinatal exposure to EFV causes structural alterations in the cytoarchitecture of the motor cortex through marked cellular loss which results in behavioral alterations and motor delay. The molecular findings in the motor cortex are similar to those in infralimbic, prelimbic and cingulate subareas of the mPFC. We demonstrate a developmental delay and persistent molecular and cellular changes in the cerebral cortex of rats exposed perinatally to EFV (Chapter 6).

Finally, in Chapter 7 we summarize the main findings and discuss the results of the studies described in Chapters 2-6.

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2

Differences in cell cycle exit and differentiation state underlie distinct timelines of mouse prefrontal and somatosensory cortex development

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Abstract

The cortex is a complex brain region organized into six layers and responsible for higher cognitive functions. Proper cortical development depends on consecutive processes, including proliferation, migration, differentiation and cell death, and follows an organizational dynamic process from early developing dorsal areas with basic functions to frontal regions with associative cognitive functions, such as the medial prefrontal cortex (mPFC). Furthermore, the cortex is sculpted by an invasion of migrating interneurons and afferents from other brain areas, each guided by intrinsic and extrinsic cues. Disturbances in cortical development have been associated with various neuropsychiatric disorders. Yet, the molecular and cellular programs controlling the development of the PFC are still poorly described. Here, we used BrdU and Ki67 assays to compare the proliferative events, such as cell-cycle length and exit, occurring during the development of murine PFC with those of a more dorsal cortical region, the somatosensory cortex (S1). We furthermore examined the expression of several transcription factors (Ctip2, Tbr1, Satb2 and Cux1) in neurons of mPFC subareas (infralimbic, prelimbic and cingulate cortex) and compared these expression profiles with those in the S1. Lastly, we studied how the serotonergic and dopaminergic systems innervate these regions over time. Altogether, the frontal areas appeared to show a delay in neuronal maturation relative to that in the more dorsal S1 area, suggesting that the PFC takes longer to mature than the S1. This is important regarding the timing of risk factors presented during neurodevelopment, which eventually may lead to disturbances in prefrontal functioning.

Keywords: Medial Prefrontal Cortex; PFC Development; Somatosensory Development; Cortex Layering; Corticogenesis

Introduction

Proper brain development requires a complex and dynamic orchestration of events that is under genetic as well as environmental control[1,2]. Cortical development involves various processes beginning with neurogenesis of neuroepithelial cells of the telencephalon (from ~ embryonic day (E) 10 to E12 in mice) and progress to stages of neural migration, differentiation, synaptogenesis, pruning and myelin formation[3,4,5]. All areas contain five or six neuronal layers which develop in an inside-out organization[1]. Newborn neurons migrate using radial glial cells as scaffolds to reach the positions within the cortical plate (CP), with the deepest cortical layer emerging first, and the later-born migratory neurons forming successively more superficial layers[6,7,8,9].

The control of complex cognitive functions, which basically involves all brain areas, is executed by the medial prefrontal cortex (mPFC)[2,10,11]. Our memory, planning, personality, emotion, decision making, inhibitory control, and attention functions are classified as executive functions, with a predominant activation of the mPFC[12]. Alterations in the maturation of the mPFC can eventually lead to higher impulsivity, aggressiveness and social deficits[13]. To unravel the complex cellular and molecular mechanisms of mPFC development, longitudinal studies are needed[1,14]. About 85% of the neuronal population within the mPFC is represented by excitatory projection neurons born in the ventricular zone (VZ) and subventricular zone (SVZ), and migrating radially to the correct position within the CP and the remaining 15% are inhibitory GABAergic neurons that arise from different progenitor zones located in the lateral and medial ganglionic eminences[15,16]. The GABAergic interneurons migrate tangentially to the mPFC area, guided by intrinsic and extrinsic factors, and reelin released by the Cajal-Retzius (CR) cells[17,18]. Around postnatal day (P) 4, all layers of the cortex are positioned[9].

When arrived at their final position, the neurons differentiate, and start to make connections with other brain areas through axon guidance molecules and their receptors[19]. Also, there is an increase in myelination and in the number and complexity of synapses[1]. Neurons that occupy the same radial positions are born in approximately the same time window and share common projection targets[20]. Deep cortical layers V and VI consist of corticofugal projection neurons and project to subcortical targets including the thalamus, internal capsule, spinal cord and tectum[21]. These neurons express, amongst others, the transcription factors COUP-TF-interacting protein 2 (Ctip2, also named Bcl11b) and T-box brain 1 (Tbr1)[22,23,24] that are important for the

specification of deep-layer neurons and subplate[25,26]. In turn, upper-layer neurons, which include layer II/III projection neurons and layer IV thalamorecipient neurons, process intracortical connections and project their axons to the ipsilateral and contralateral cortex, and are marked by the expression of the transcription factors cut-like homeobox 1 (Cutl1 or Cux1) as well as special AT-rich sequence-binding protein 2 (Satb2)[25,27,28].

As development proceeds, the mPFC furthermore receives innervations from various neural systems in the developing brain, such as the cholinergic, catecholaminergic and monoaminergic systems[29,30]. The integration of these neurotransmitter signals in the mPFC contributes to development, regulating various events during corticogenesis, including the formation of appropriate connections and resulting behavior[31]. Around E10, cholinergic projections originating from neurons located in the basal forebrain, distributed between the pedunculopontine and the laterodorsal tegmentar nuclei, innervate the cerebral cortex, and promote neuronal maturation and plasticity[32]. Around the same time dopaminergic and serotonergic neurons located in the ventral tegmental area (VTA)/substantial nigra (SN) and dorsal raphe nucleus (DRN), respectively, send their projections to the frontal cortical areas[33-37]. These projections innervate their cortical targets approximately around E14 during the peak of migration of GABAergic interneurons, but only at E16 they reach the mPFC subareas[37,38,39]. These axons initially are distributed in the marginal zone (MZ) and below the subplate (SP)[40,41]. Serotonin (5-HT) and dopamine (DA) are important neurotransmitters and neurotrophic factors that can control proliferation, migration, differentiation and cell death by directly binding to their receptors that are expressed on various cortical cell types or indirectly via CR cells, present in the MZ[39,41,42,43]. Around E12, axonal projections of subpopulations of norepinephrine (NA) neurons, located in the brainstem, begin to project towards the frontal areas[44]. NA plays a role in the organization and function of neuronal circuit formation and the synaptic plasticity of astrocytes and microglia[44,45]. Together, this indicates that there is ample evidence for the interaction of multiple neuronal systems during cortical development.

Cortical specification of visual, sensory, motor, associative and cognitive areas is regulated by a tightly controlled expression of transcription factors (intrinsic factors) as well as guidance cues (extrinsic factors), sculpted by environmental experiences[46]. Brain maturation and connections between different areas do not occur homogeneously or in the same time window, but follow a specific sequence with a characteristic directional path[47,48].

However, regional maturation is more related to the function exerted by that specific area than to its location, such that areas with more complex functions ultimately develop following the development of the primitive functions[46,47,48]. The last regions of the brain to fully mature are the prefrontal, orbitofrontal and upper temporal cortical areas involved in complex executive functions and multimodal integration[47,48,49,50]. Because the mPFC is one of the last to develop and integrates inputs from various other brain areas into more complex functions, this relatively long developmental window makes the mPFC most vulnerable to all kinds of risk factors. This can eventually result in a psychiatric illness associated with the occurrence of various behavioral disturbances[51,52]. The dorsolateral PFC, and the infralimbic, prelimbic and cingulate cortex of the mPFC are regions that are often affected in autism spectrum disorder (ASD), schizophrenia, depression and anxiety disorders[53,54]. Therefore, it is important to perform detailed longitudinal studies on how the mPFC develops.

The purpose of this study was two-fold. As the mPFC consists of a conglomerate of subareas, we 1) compare the development of each of these subareas with one another and with the hallmark of corticogenesis, the well-described primary somatosensory cortex (S1), and 2) examine whether the mPFC matures differently and/or in an extended manner compared to the more dorsal S1 region. To these ends, we analysed the proliferation status and cell-cycle exit events, studied the expression of the transcription factors Satb2, Ctip2, Tbr1 and Cux1, and evaluated the serotonergic and dopaminergic innervation of the mPFC subareas in relation to S1.

Methods

Animals

All animal use and care were performed in accordance with the institutional and national guidelines and regulations of the Committee for Animal Experiments of the Radboud University Nijmegen, The Netherlands. Wild-type C57Bl/6 mice were obtained from Harlan Laboratories B.V. (The Netherlands) and housed with *ad libitum* food and water access, with a 12 hr light cycle at a controlled ambient temperature (21 ± 1°C) and a 60% relative humidity. Matings were set up late in the afternoon and vaginal plugs checked early in the morning to lower the variability between developmental time points. Pregnancy was confirmed after a vaginal plug was found and was considered E0.5. Pregnant mice were individually housed in standard

Macrolon® cages and sacrificed by means of cervical dislocation and the embryos collected at E14.5, E16.5 and E18.5 and pups were decapitated at P7.

Tissue treatment and immunohistochemistry

Brains were rapidly dissected, collected in phosphate-buffered saline (PBS, pH 7.4) and fixed for 30-90 min by immersion in 4% paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS. After fixation, brains were washed in PBS, cryoprotected in 30% sucrose (Sigma-Aldrich, St. Louis, Missouri, United States) in PBS, frozen in M-1 embedding matrix (Thermo Fisher Scientific, Waltham, MA, USA) on dry ice in a plastic cup and stored at -80°C. Cryostat coronal or sagittal sections were cut at 16 µm, mounted in series of 6–8 on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at -20°C. Cryosections were stained immunohistochemically as described previously (Kolk et al., 2006). Primary antibodies used included mouse anti-Cux1 (1:300, Abcam, ab54583, Cambridge, United Kingdom), rat anti-Ctip2 (1:500, Abcam, ab10465, Cambridge, United Kingdom), mouse anti-Satb2 (1:500, Abcam, ab5102, Cambridge, United Kingdom), rabbit anti-Tbr1 (1:500, Abcam, ab31940, Cambridge, United Kingdom), mouse anti-NeuN (1:500, Merck Millipore, MAB377, Bedford, MA, USA), rabbit anti-5-hydroxytryptamine (5-HT) (1:500, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands, S5545) and rabbit anti-tyrosine hydroxylase (TH) (1:500, Merck Millipore, AB152, Bedford, MA, USA). Antibodies were diluted in blocking buffer (BB) with 1.7% normal donkey serum, 1.7% normal goat serum and 1.7% normal horse serum, 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, Missouri, United States), 1% glycine (Sigma-Aldrich, St. Louis, Missouri, United States), 0.1% lysine (Sigma-Aldrich, St. Louis, Missouri, United States) and 0.4% Triton X-100 in PBS and incubated overnight at 4°C. Secondary antibodies included Alexa 488 goat anti-mouse, Alexa 555 goat anti-rat, and Alexa 568 goat anti-rabbit (Molecular Probes, Life Technologies, Grand Island, NY at 1:500) and diluted in BB for 60 min at room temperature (RT). After washing in PBS, sections were counterstained with fluorescent Nissl stain (1:500, NeuroTrace; Invitrogen) or DAPI (1:1000, Molecular Probes) diluted in PBS for 15 min, thereafter, washed extensively in PBS and embedded in 90% glycerol in PBS. For visualization, a Leica DMRA Fluorescence microscope with DFC340FX camera and LASAF software or a Leica DMI6000B automated high-content microscope were used. Figure

images were reconstructed and digitized using Adobe Photoshop CS6 and Adobe Illustrator CS6.

Analysis of cell-cycle entrance and exit

To label proliferating cells, pregnant mice received an intraperitoneal injection of 5-Bromo-2-deoxyUridine (BrdU, BD Pharmingen, San Diego, 10 mg/mL in PBS) at E14.5, E16.5, and E18.5 in a dose of 50 μg/g body weight. The animals were either sacrificed after 2 hours (proliferation paradigm) or 24 hours (migration paradigm) following treatment with BrdU by cervical dislocation, and brains were rapidly dissected and processed for fixation, freezing, and cryosectioning as described above. We incubated the sections for 12 seconds in 1:2 trypsin solution (0.1% trypsin, 0.1% $CaCl_2$ in 0.1M Tris buffer). Trypsin was inactivated with fetal bovine serum (FBS) for 10 minutes, followed by a wash in PBS and incubation in pre-warmed 2N HCl solution for 30 min at 37˚C. The solution was neutralized by incubating in 0.1 M sodium borate buffer (pH 8.5) for 5 min at RT followed by wash in PBS for 30 minutes. Next, immunostaining was performed as described above using mouse anti-BrdU (1:500, Novus Biological, NB500-439) and rabbit anti-Ki67 (1:500, Abcam, Cambridge, United Kingdom, ab16667) overnight. The secondary antibodies included Alexa-conjugated 488 goat anti-mouse and goat anti-rabbit 555 (Molecular Probes, Life Technologies, Grand Island, NY at 1:500) and were diluted in BB for 30 min at RT.

Data analysis

For assessing the number of Satb2-, Ctip2-, Cux1-, Tbr1- and NeuN-positive cells, at least four to seven mouse embryo/pup brains were analysed for each marker, and three to six consecutive sections per brain were quantified. BrdU-positive cells were counted per animal (n = 3-4) and expressed as a percentage of proliferative activity in total $Nissl^+$ cells. For cell cycle length and exit analysis, sections were double labeled with BrdU and the cell proliferation marker (Ki67). For the cell cycle length assessment, Ki67-positive ($Ki67^+$) cells and Ki67- and BrdU-double positive ($Ki67^+/BrdU^+$) cells were counted and the proportion of $Ki67^+/BrdU^+$ cells to the total number of $Ki67^+$ cells was calculated. For the cell cycle exit analysis, $BrdU^+/Ki67^+$ cells and $BrdU^+/Ki67^-$ cells were counted and the proportion of $BrdU^+/Ki67^-$ cells (cells withdrawn from the cell cycle) to the total number of $BrdU^+$ cells was calculated. A 0.1-mm-wide rectangle spanning the

prefrontal wall was placed over the center of the subarea (either infralimbic, IL; prelimbic, PL; or cingulate cortex, CG) of the mPFC and S1. The overall cortical length of a subarea was divided into ten equal bins [bin 1 within the deep cortical layers and bin 10 within the presumptive layer I] within this rectangle, and 5-HT- or TH-positive axon length or number of marker-positive neurons were counted within each bin using ImageJ software including the NeuronJ plugin (NIH, Bethesda, USA). Data were normalized to a total length per bin or to a percentage of the total number of $Nissl^+$ or $DAPI^+$ cells and averaged for each animal.

Statistical analysis

Depending on whether data fulfilled the assumptions of homogeneity of variance and normality, data were analyzed either by a Student T-test, one-way ANOVA ($\alpha = 5\%$) or Mann–Whitney U test followed by Tukey posthoc testing using GraphPad Prism 6 (San Diego, CA) or Excel data analysis toolkit (Microsoft Office) and expressed as means ± SEM.

Results

Radial cortical expansion differs between PFC and S1

The pre- and postnatal assessment of cortical thickness can be used to identify functionally distinct cortical areas[55,56]. The thickness and the overall cell density of subdomains of the mPFC and S1 were measured in coronal sections labelled with Nissl or DAPI at E14.5, E16.5, E18.5, and P7. At E14.5, a significant increase in cortical thickness was found in the S1 when compared with PL ($p = 0.00004$) and CG ($p = 0.014$) but not with IL ($p = 0.286$) (Fig. 1C). However, no differences were found in the total number of cells per area when comparing S1 with IL (Fig. 2B; $p = 0.327$), PL (Fig. 2C; $p = 0.900$) and CG (Fig. 2D; $p = 0.362$). At E16.5, cortical thickness in S1 was significantly higher compared to IL ($p = 0.00002$) and PL ($p = 0.00005$), but not to CG ($p = 0.223$) (Fig. 1D). However, a higher number of cells was found in VZ/SVZ and IZ in the frontal areas when compared with S1 (Fig. 2E; bin3, $p = 0.047$; bin4, $p = 0.014$; bin5, $p = 0.034$), PL (Fig. 2F; bin3, $p = 0.036$; bin4, $p = 0.006$; bin5, $p = 0.007$; bin6, $p = 0.0008$), and CG (Fig. 2G; bin1, $p = 0.032$; bin3, $p = 0.047$; bin7, $p = 0.015$). At E18.5, a significant increase in the thickness of the CG was found when compared with S1 (Fig. 1E; $p = 0.000005$), but in the other prefrontal subareas the thickness of the S1

was significantly larger (Fig. 1E; IL, p = 0.00001; PL, p = 0.00001) with a significantly higher number of cells in the CP of S1 when compared with IL (Fig. 2H; bin10 p = 0.054), PL (Fig. 2I; bin6, p = 0.0005; bin10, p = 0.001) and CG (Fig. 2J; bin6, p = 0.021; bin10, p = 0.015). A similar pattern was found at P7, where only the thickness of the GC was significantly larger than that of the dorsal S1 (p = 0.004) (Fig. 1F), but the number of cells in the S1 was significantly higher in upper-layers when compared with IL (Fig. 2K; bin8, p = 0.002; bin9, p = 0.001) and PL (Fig. 2L; bin9, p = 0.005) and in all layers of the CG (Fig. 2M; p = 0.003). Together, the results suggest an early temporal development in the S1 at the level of cortical thickness when compared with mPFC subareas, such as in cell density in the CP, where post-migrational cells are located.

Prefrontal subregions show a delay in proliferation rate compared to the S1 region

To investigate whether the mPFC subareas show differences in cell division compared to the S1, we performed at the same developmental age BrdU experiments in combination with immunohistochemical localization of Ki67. BrdU incorporates during the S-phase of the cell cycle and Ki67 is a marker for proliferating cells in all stages of the cell cycle, but is absent in resting cells (G0) and differentiated cells[57,58,59]. To assess relative changes in mPFC and S1 cell-cycle length, we calculated the percentage of $BrdU^+/Ki67^+$ cells relative to the total number of $Ki67^+$ cells 2 hours after BrdU injection (Fig. 3A-G). First, we compared the number of proliferating cells between the cortices. Significant percentages of $BrdU^+$ cells were found in the VZ/SVZ of the IL (Suppl. Fig. 1A; bin5, p = 0.00002, bin6, p = 0.0001, bin7, p = 0.023), the PL (Fig. 3B; Suppl. Fig. 1B; bin5, p = 0.001, bin6, p = 0.0001, bin7, p = 0.006, bin8, p = 0.046) and CG (Suppl. Fig. 1C; bin5, p = 0.001, bin6, p = 0.002, bin7, p = 0.009, bin8, p = 0.045) cortices when compared to the S1 at E14.5 (Fig. 3B). When we analysed the percentage of $Ki67^+$ cells, no differences were found between the IL (p = 0.67) and PL (p = 0.47) subareas compared to the S1 (Fig. 3B), although we found a difference in the CG (p = 0.02) compared to the S1 (Fig. 3B). A significant number of proliferative cells was found in $BrdU^+/Ki67^+$ cells in the frontal subareas IL (Fig. 3E; bin4, p = 0.029, bin5, p = 0.0003, bin6, p = 0.0004), PL (Fig. 3F; bin5, p = 0.00001, bin6, p = 0.00002, bin7, p = 0.0008, bin8, p = 0.041) and CG (Figure 3G; bin2, p = 0.038, bin5, p = 0.0001, bin6, p = 0.0001, bin7, p = 0.011; bin8, p = 0.003) when compared with the S1. At E16.5, we did not find significant

differences between the expression of the markers in all subareas examined, namely IL (BrdU, p = 0.287 (Fig. 3C; Suppl. Fig. 1D); Ki67, p = 0.488; double labelling, p = 0.678; Suppl. Fig. 2A), PL (BrdU, p = 0.585 Fig. 3C; Suppl. Fig. 1E); Ki67, p = 0.916; double labelling, p = 0.84; Suppl. figure 2B) and CG (BrdU, p = 0.187 (Figure 3C; Suppl. Fig. 1F); Ki67, p = 0.317; double labelling, p = 0.082, Suppl. Fig. 2C) compared to the S1. At E18.5, following 2h of BrdU exposure, we did find significant difference in the number of proliferative cells only when comparing CG subareas with the S1 (Fig. 3D, p = 0.0017; Suppl. Fig. 1G-I; Suppl. Fig. 2D-F). Next, the balance between cell-cycle re-entry and exit was determined using BrdU/Ki67 immunolabeling 24 h following BrdU injection (Fig. 3H-O). Cells that re-entered the cell cycle at 24 h were $BrdU^+/Ki67^+$, while those that exited were $BrdU^+/Ki67^-$ [60]. At E15.5, no significant differences were found in the percentage of cells that re-entered the cell cycle between S1 and IL (Fig. 3N; p = 0.84), PL (Fig. 3N; p = 0.57) in superficial domains but not in CG (Fig. 3N; p = 0.24). Instead, in CG more cells were found to exit the cell cycle ($BrdU^+/Ki67^-$) when compared with S1 (Fig. 3L; p = 0.014). At E17.5, we did not find significant differences in the percentage of positive cells that exited the cell cycle between S1 and the frontal areas IL (p = 0.826), PL (p = 0.745) and CG (p = 0.801) (Fig. 3M). Significant differences in the percentage of cells that re-entered the cell cycle in S1 when compared with IL (p = 0.004), PL (p = 0.00001) and CG (p = 0.002) were found (Fig. 3O). Together, the data suggest that early in development there are more cells that re-entered the cell cycle in frontal areas when compared to S1, but this difference is not maintained after 24h exposure, when more cells were found to re-enter the cell cycle in the dorsal area when compared with mPFC areas, suggesting a delay in frontal development.

Developmental differences in the deepest cortical layer between PFC subareas and the S1

Tbr1 seems to play distinct roles at different stages of cortical development. At early stages, Tbr1 promotes a frontal identity while suppressing caudal identity[61]. The $Tbr1^+$ cells derived from the first progenitor cells are located in layer VI[62] and contribute to the development of corticothalamic projection neurons[62,63], but not sub-cerebral projection neurons[64]. To distinguish between these cells, we labeled sections of the cortical brain with anti-Tbr1 antibody and analysed the $Tbr1^+$ cells per area expressed in subareas of the mPFC and S1. The expression of Tbr1 was

comparable between the frontal and sensory areas at E14.5 and decreased during further development (Fig. 4A-E). Tbr1 expression was high at E14.5 in CP but was not significantly different when comparing S1 with IL (Fig. 4F, p = 0,391), PL (Fig. 4G, p = 0,561) and CG (Fig. 4H, p = 0,684). At E16.5, the expression of Tbr1 in CP and SP was higher in the IL (Fig. 4I, bin10, p = 0,005), PL (Fig. 4J, bin6; p = 0,003) and CG (bin6; p = 0.0002) cortices when compared to S1. In contrast, cells from the CG expressed less Tbr1 and the peak of expression was found at E18 (Fig. 4D and N). At E18.5, the number of $Tbr1^+$ cells per area increased significantly in intermediate zone (IZ) and SP of S1 compared to IL (Fig. 4L, bin4, p = 0.041; bin5, p = 0.026), PL (Fig. 4M, bin4, p = 0.012; bin5, p = 0.000004, bin6, p = 0.001) and CG (Fig. 4N, bin4, p = 0.012; bin5, p = 0.0001), but significantly less in the CP in which more $Tbr1^+$ cells were found in IL (bin8, p = 0,004; bin9, p = 0,001), PL (bin8, p = 0,053; bin9, p = 0,004) and CG (bin8, p = 0,001; bin9, p = 0,0007) (Fig. 4L-N). At age P7, there was a decline in all cortical areas examined. A significantly higher number of $Tbr1^+$ cells per area was found in deep layers in IL (Fig. 4O) and PL (Fig. 4P) compared to S1, but not in the upper-layers, and in CG and S1 (Fig. 4Q). These data suggest that deep-layer development was initiated approximately at the same time and place when the frontal and dorsal cortical areas were compared. At the peak of expression at E18 frontal subareas showed more $Tbr1^+$ cells in the more superficial aspects of the CP when compared to the S1, although almost comparable levels at P7. This is indicative for a developmental delay.

Satb2 expression during cortical development

Following the corticogenesis steps, we analysed the next layer to arise, layer V. One potential regulatory determinant of this type of cells is Satb2, which is expressed during differentiation in a subgroup of neurons in layers II to V[65]. Consistent with previous reports[66], we detected Satb2 immunoreactive cells in the CP at E14.5 in all cortical areas, but this was not significantly different when comparing S1 with IL (p = 0.486), PL (p = 0.767) and CG (p = 0.964) (Fig. 5F-H). At E16.5, when late-born neurons are predominantly migrating, the number of $Satb2^+$ cells increased significantly in CP in IL (Fig. 5I, bin7, p = 0.005) and PL (Fig. 5J, bin7, p = 0.004), and SP in CG (Fig. 5K, bin6, p = 0.04) when compared with S1. Also at E18.5, an increase of $Satb2^+$ cells in all cortical areas was noted, which was significant in the CP and layer VI in S1 compared to IL (Fig. 5L, bin8, p = 0.0004) and PL (Fig. 5M, bin5, p = 0.0009; bin6, p = 0.033) and in IZ and

layer VI in CG (Fig. 5N, bin4, p = 0.039; bin5, p = 0.008; bin8, p = 0.040). Instead, a significant number of $Satb2^+$ cells were found in CP of the CG when compared with S1 (Figure 5N, bin9, p = 0.024; bin10, p= 0.042). The expression of Satb2 was strongly reduced at P7 in the superficial layers, as demonstrated by the reduction in the number of $Satb2^+$ cells in the subareas of the mPFC and the S1 (Fig. 5O-Q), when the cortical neurons ended the migration and are refining their axonal projections. We found a significant increase of $Satb2^+$ cells in upper-layers of S1 when compared with IL (Fig. 5O, bin8, p = 0.008; bin9, p = 0.002), PL (Figure 5P, bin9, p = 0.020) and CG (Figure 5Q, bin7, p = 0.005; bin8, p = 0.003). Interestingly, at E14.5 and E16.5 no $Satb2^+$ cells were found in active proliferation zones, including the SVZ and the underlying VZ, and only a limited number of $Satb2^+$ cells in the IZ at E18.5 in both the frontal and dorsal areas (Fig. 5A, F-K). These data suggest that Satb2 is not expressed by cortical neurons prior to migration and that temporal expression seems to be higher at E16.5 in IL and PL when compared to S1, demonstrating more cells in the layer V. The peak of expression of this marker in all evaluated areas appears to be at age E18.5. We identified a significantly higher levels of Satb2 expression in S1 cortex when compared to the frontal areas, especially in the superficial layers. In addition, the expression of this marker seems more delayed in the cortex CG cortex when compared with other frontal areas and S1. Together, these findings suggest a delay in the frontal area development when compared with that of S1.

Patterned expression of Ctip2 in neocortical layers II–V

In the cortical brain, Ctip2 is used as a marker for a subset of subcerebral projection neurons of deep layer V, as well as GABAergic interneurons, but is also expressed in other cell types in layers II, III and IV[26,67]. Immunohistochemistry revealed that during the peak production of the deep layer neurons between E12.5 and E14 the onset of Ctip2 expression in the CP is restricted to post-migratory neurons without expression in VZ or in migrant neurons of IZ (Fig. 6A, F-H), without significant differences in expression at E14.5 between cortical areas when comparing S1 with IL (p = 0.836), PL (p = 0.901) and CG (p = 0.409). At E16.5, when surface layer neurons are generated, Ctip2 is strongly expressed in the CP (Fig. 6A, I-K). There was a significant increase of $Ctip2^+$ cells in CP in IL (Fig. 6I, bin6, p = 0.00003; bin7, p = 0.00001; bin8, p = 0.018), PL (Fig. 6J, bin7, p = 0.0003; bin8, p = 0.014) and CG (Fig. 6K, bin9, p = 0.002) when compared with S1.

At E18.5, all mPFC subareas showed a significantly higher level of Ctip2$^+$ cells per area when comparing to S1 (IL ($p = 0.0006$), PL ($p = 0.000008$) and CG ($p = 0.0001$) (Fig. 6B-E, L-N)). On P7, significant down-regulation of Ctip2 starts in all cortical areas, with the IL having a significantly higher number of Ctip2$^+$ cells in deep-layer (Fig. 6O, bin1, $p = 0.018$; bin2, $p = 0.020$, bin5, $p = 0.003$) when compared with S1 but not in superficial layer cells, where we found more expression in S1 when compared with IL (Fig. 6O, bin9, $p = 0.0009$; bin10, $p = 0.002$). The same pattern was observed in superficial layers of PL (Fig. 6P, bin9, $p = 0.003$; bin10, $p = 0.008$) and CG (Fig. 6Q, bin9, $p = 0.007$) when compared with S1. Together, these results suggest a common onset of Ctip2 expression at E14.5, and expression became more pronounced in frontal areas at E16.5 and E18.5 but was decreased by P7.

Expression of Cux1 during superficial layer organisation

Homeobox transcription factor Cux1 is involved in embryonic patterning and cell-type specification[68]. Cux1 is a marker for upper-layer cells and expressed in precursor cells at the time the upper-layer neurons are generated (E13-E17), but a few scattered neurons in the deep layers (V–VI) and CR cells in the adult cortical brain also express Cux1[68,69]. At E14.5, most cells in the CP represent neurons that are destined to form the deep cortical layers, and only a few of these cells also demonstrate Cux1 immunoreactivity in all sub-areas of mPFC and S1, but not significantly different when comparing S1 with IL (Fig. 7A and F; $p = 0{,}208$), PL (Fig. 7A and G; $p = 0{,}967$), and CG (Fig. 7A and H; $p = 0{,}796$). At E16.5, when a large proportion of newborn upper-layer neurons are migrating away from proliferative areas, many Cutl1$^+$ cells migrate through the IZ and the CP (Fig. 7A, I-K) being significantly higher in the IL (Fig. 7I; $p = 0.001$) and PL (Fig. 5J; $p = 0.003$) subareas when compared with S1, but not in CG when compared with S1 (Fig. 7K; $p = 0.760$). Notably, Cux1-positive cells in the CP show much more intense immunoreactivity than the VZ and SVZ cells (Fig. 7A), as also observed at E18.5 where a significant number of Cux1$^+$ cells are located in the CP of IL (Fig. 7L; $p = 0.005$), PL (Fig. 7M; $p = 0.010$) and CG (Fig. 7N; $p = 0.0001$) when compared to S1. At P7, Cux1$^+$ cells were identified in deep and superficial layers, being significantly higher expressed in superficial layers of S1 when compared with IL (Fig. 7O; bin8, $p = 0.010$; bin9, $p = 0.00001$), PL (Fig. 7P; bin9, $p = 0.003$) and CG (Fig. 7Q; bin7, $p = 0.034$; bin8, $p = 0.005$; bin9, $p = 0.011$). Therefore, at E14.5 the expression of Cux1

was not different between the frontal and dorsal areas, while at E16.5 and E18.5 there was a significant increase in expression in mPFC subdomains when compared with S1. At P7, a significantly higher number of $Cux1^+$ cells were found in S1 when compared with mPFC subareas. Thus, Cux1 marks an initial step in the acquisition of neuronal identity of the upper layer, is expressed in both the precursor population and the neurons of the upper layer, and its expression in mPFC subdomains is always higher when compared with S1.

Superficial domain *vs* deep domain : double labelling of Satb2 and Tbr1

Cortical neurons project to specific targets located nearby in the cortex or in distant subcortical regions[26,28]. Different populations of cortical neurons can be identified by their time of origin, laminar localization, projection pattern and molecular signatures, all of which are controlled by transcription factors[69]. Double marking identifies specific types of cells and give clues to which region that they will send their projections. For example, the co-expression of Satb2 and Tbr1 is required for the specification of callosal neurons, as Satb2 promotes the expression of Tbr1 in the upper-layer neurons[28,69]. When expressed alone, Tbr1 is found only in corticothalamic neurons. In order to elucidate differences in identity, we compared the developmental time-line of these double-labeled cells of the mPFC subdomains with that of the S1. At E14.5, no significant differences were found in the total number of $Satb2^+/Tbr1^+$ cells per area in IL (Fig. 8C; $p = 0.705$), PL (Fig. 8D; $p = 0.520$) and CG (Fig. 8E; $p = 0.885$) when compared with S1. At E16.5, the peak of late-born neurons, we found a significant increase in the number of double-labelled positive cells in IL (Fig. 8F; $p = 0.010$) and PL (Fig. 8G; $p = 0.019$) when compared with S1, but not in CG (Fig. 8H; $p = 0.612$). At E18.5, a higher number of these double-labelled cells was identified in the VZ and IZ in S1 compared to IL (Fig. 8I; bin4, $p = 0.013$), PL (Fig. 8J; bin4, $p = 0.015$, bin5, $p = 0.00003$) and CG (Fig. 8K; bin5, $p = 0.002$). In contrast, a significant increase of $Satb2^+/Tbr1^+$ cells in the CP in IL (bin9, $p = 0.005$) and CG (bin8, $p = 0.017$; bin9, $p = 0.001$) was found when compared with S1 (Fig. 8I and K). Seven days after birth, there was a significantly higher number of double-positive cells in upper-layer neurons in IL (Fig. 8L; $p = 0.02$) and PL (Fig. 8M; bin6, $p = 0.02$) when compared with S1, except in superficial layers where we found more positive cells in S1 when compared with IL (bin8, $p = 0.03$; bin9, $p = 0.0008$) and PL (bin9, $p = 0.003$) (Fig. 8L and M). No differences were found in CG when

compared with S1, except in the most superficial layer (Fig. 8N; bin10, p = 0.039). Together, the data suggest that the callosal projection neurons appear at the same time point in frontal and dorsal areas in the cortex, however at E16.5 the CG showed a delayed expression when compared with the dorsal region.

Differences between frontal and dorsal areas during cortical maturation

In order to elucidate the maturation state of the prefrontal subdomains compared to the S1, we stained for the neuronal nuclear protein (NeuN), localized in nuclei and perinuclear cytoplasm and considered to be a unique marker of postmitotic neurons[70]. In mice, NeuN expression starts around E16, and peaks at E18.5 and P7[9]. At E18.5, we did not find significant differences in the number of $NeuN^+$ neurons between S1 and IL (Fig. 9A and B; p = 0.499), PL (Fig. 9C; p = 0.853) and CG (Fig. 9D; p = 0.822). However, at P7 the neuronal density in all cortical layers of the S1 was significantly higher when compared to IL (Fig. 9E and I; p = 0,002; bin2, p = 0.008; bin3, p = 0.003; bin8, p = 0.001; bin9, p = 0.00007; bin10, p = 0.0028), PL (Fig. 9F and I; p = 0,001; bin3, p = 0.01; bin7, p = 0.006; bin8, p = 0.006; bin9, p = 0.00002; bin10, p = 0.00008) and CG (Fig. 9G and I; p = 0,0004; bin2, p = 0.0006; bin3, p = 0.00002; bin4, p = 0.002; bin5, p = 0.007; bin6, p = 0.03; bin7, p = 0.002; bin8, p = 0.0010). Together, the data suggest a delay in maturation of the mPFC when compared to somatosensory cortical maturation.

Serotonergic and catecholaminergic innervation of the mPFC and S1

A proper cortical development requires extrinsic signals that include monoaminergic and catecholaminergic signals and growth factors. Together they regulate a series of timed events, which include proliferation, migration (via contact with the CR cells) and differentiation of neurons (i.e., proper growth and orientation of axons towards their targets), and occur mainly during embryonic and postnatal development[3,71-75]. In particular, 5-HT and DA are important regulators of cortical development[30,36,37,39]. In mice, serotonergic and dopaminergic neurons send their projections to frontal areas around E10-E11 and reach the PFC around E14.5[39]. We analyzed the total length of $5\text{-}HT^+$ fibers in all subdomains of the mPFC and the S1 at E18.5 when these fibers are more distributed throughout all cortical structures (Fig. 10A-E). There was a significant increase in the lengths of these fibers in deep as well as superficial domains of the IL (Fig. 10C; bin1, p = 0.005; bin2, p =

0.026; bin3, p = 0.0004; bin4, p = 0.012; bin8, p = 0.00001), PL (Fig. 10D; bin2, p = 0.008; bin8, p = 0.0005) and CG (Fig. 10E; bin2, p = 0.0007; bin4, p = 0.012) when compared with the S1. The same pattern was found for dopaminergic innervation, where a significant increase in the mean lengths of TH^+ fibers was found in the IL (Fig. 10F; bin3, p = 0.002; bin4, p = 0.007; bin7, p = 0.002), PL (Fig. 10G; bin7, p = 0.01) and CG (Fig. 10H; bin2, p = 0.03; bin3, p = 0.019; bin6, p = 0.017; bin7, p = 0.017; bin10, p = 0.018) when compared with the S1. Together, the data showed a higher innervation of 5-HT and DA in the frontal areas when compared with a dorsal area.

Discussion

Our objective was to investigate the similarities and differences in the development and maturation of the mPFC subdomains and the S1. When evaluating the cell cycle, a higher degree of proliferation in the frontal areas at the beginning of development was observed, when compared to S1. This difference does not persist after 24h, when more cells continued to proliferate in the dorsal area when the mPFC subareas were exiting the cycle. Through transcription factor expression analysis of the organization of the cortical layers, we identified a common starting point that was comparable between the subareas of the mPFC and S1. Both the deep layers, marked with Tbr1, Satb2 and Ctip2, as well as the upper layers, labeled with Cux1, showed the highest expression in the subareas of mPFC when compared to S1. In addition, each marker presented a unique expression pattern in the cortical domains analysed. Using NeuN as a marker, we found a delay in the maturation of the mPFC subdomains when compared to the S1. Finally, when evaluating serotonergic and dopaminergic innervation, using 5-HT and TH as markers, respectively, we found that the mPFC subareas required a higher innervation of these systems when compared to the S1. Altogether, our findings suggest that the development of the mPFC areas was delayed and demanded a high expression of various transcription factors, which may be associated with their higher complexity when compared to the S1.

The neocortex occupies about 80% of the total human brain volume and is the most recent brain structure from an evolutionary point of view[1,76,77]. The PFC develops from neuroepithelial germ cells and proliferates, from E11 to E12 in mice, to form a vast heterogeneous population of cells[3]. Using cross-sectional magnetic resonance imaging studies on the brain, the development of the human cortex has been quantified by measuring the gray matter density in each lobe[80]. The first areas to mature are those with the most basic

functions, for example brain areas that process sensibility and movement[81]. Thus, the older cortical areas mature earlier than the new associative cortices that integrate information[80-,84]. The PFC has more advanced functions, including the integration of sensory, motor, reasoning and other executive functions, and is the last to mature in early adulthood[46,85,86]. In mice, we found a similar pattern of development with faster maturation of sensory and motor regions when compared to frontal areas, in line with results from other studies[87,88]. During development, proliferative cells are distributed throughout the brain and cell density progressively increases from the dorsal region of the brain to the frontal region[78,79]. We reconstructed this progenitor distribution in the embryonic brain using BrdU and Ki67 labeling, and identified a higher proliferative activity in the prefrontal subareas compared to the S1, but 24h after the BrdU injection the S1 contained more cells reentering the cell cycle. In general, regional cortical thickness is similar until early postnatal life compared to those observed in older children and adults[75,77], with the cortex reaching the final thickness in late adolescence and early adulthood[83,89]. In addition to a larger number of cells and mature neurons, at the early postnatal age we found a thicker somatosensory cortex when compared to the mPFC subareas. During this period of brain development, the dorsolateral PFC and mPFC expand almost twice as much as some other regions, including the occipital and medial insular cortex[47,100-102]. Throughout childhood and adolescence, the development of the brain is characterized by expansion followed by a decline of gray matter volume and increase in the volume of white matter[102,103]. Significant changes in cortical thickness with varying gray- or white-matter volumes are commonly found in developmental disorders such as bipolar disorder, attention deficit / hyperactivity disorder (ADHD), schizophrenia[104] and Williams syndrome[80]. Studies focusing on cortical thickness are important for the diagnosis of brain disorders and accompanying cognitive problems, and to establish whether the disorder is stable or progressive[80].

Correct cortical development depends on stimuli from external brain areas, via neurotrophic and neurotransmitter signaling. Some of these afferents responsible for the control of cell proliferation, migration and apoptosis arise from the serotoninergic system[37,39,40]. In addition to the initial events of corticogenesis, such as proliferation, migration, layer organization and apoptosis, the action of 5-HT as a developmental neuromodulator involves the adjustment of neuronal excitability via the various 5-HT receptors that are expressed in a spatial and temporal manner[30,35,36]. Serotonergic fibers emerge from the raphe nucleus while the dopaminergic

fibers emerge in the midbrain, both around E10.5[41,74]. The ascending 5-HT and DA fiber passages are visible at E12, running on both sides of the midline, at which point the 5-HT axons leave the main tract of the medial forebrain bundle and begin to follow other fascicles until they arrive in the frontal areas[41,36]. At E14.5, these fibers innervate the somatosensory and frontal cortex, but only around E16.5 they are visible in the mPFC subareas[39,74]. These fibers target different cell types, for example, CR cells, astrocytes and neurons that express various receptors for 5-HT and DA[36,105], thus actively participating in cortical development. A higher prefrontal than somatosensory cortex innervation by 5-HT and DA fibers was found in our study, which may be explained by the higher integrative complexity of the frontal than the somatosensory cortex. In rodents, changes in serotonergic signaling result in abnormal barrel formation, a reduction in cortical thickness and expression of activity-regulated genes essential for the formation of inhibitory cortical networks[106] in the somatosensory cortex[107,108]. Animals lacking the 5-HT transporter showed an increase in serotonergic innervation of the mPFC and a decrease in the number of $Satb2^{+}$ cells[109], as well as a smaller number of CR-cells. These changes resulted in a cortical disorganization which interfered with cortical function and behaviour later on[109,41], supporting an important role of 5-HT in mPFC developmental processes.

In the quest to understand which factors contribute to the normal course of brain development and to neurodevelopmental disorders, genetic and imaging methods are combined to answer questions about the influence of genes and the environment on brain structure[21,22,110]. However, little is known about the mechanisms that control the specification of neurons in different cortical layers, whereby transcription factors are responsible for the early brain developmental events[22,26,111]. Neurons of layer IV emerge around E14, and layers II and III neurons around E15 and E16[26,112]. Tbr1 and Ctip2 are associated with the specification of neurons in the deep layers VI and V, respectively[66], while Cux1 and Satb2 are expressed in the initial stages of the specification of the neurons of the upper layers[15,66,68].

In the neocortex, Tbr1 mRNA is expressed in postmitotic cells of the developing cortex from E10 to adulthood, with particularly high preplate expression and lower expression in the IZ, and no expression has been detected in the VZ[15]. Similar to our results in the mPFC and S1, the peak of Tbr1 expression appears to be from E16 to E18 and is more pronounced in the S1. The cortex of mutant Tbr1 mice shows developmental abnormalities in the laminar organization of neurons and orientation of afferent and efferent cortical axons, making these mice a suitable animal model for

autism[61,62,113,114,115]. Ctip2 is expressed at high levels in layers IV and V in a pattern that is comparable between cortical areas, the ventrolateral cortex as well in the ventromedial cortex. At E12, when the initial cortical progenitors are dividing, Ctip2 is expressed only in a small cluster of cells in the ventrolateral cortex[66,116]. At E14 and E16, during the peak of corticospinal motor neuron (CSMN) production, Ctip2 is highly expressed by post-mitotic cells in the CP, but not by cells in the VZ or SZ in the S1, suggesting that expression of this marker starts in the postmitotic CSMN[116]. Mice mutant for Ctip2 are born alive but soon die, showing the important function of this gene during early development[22, 117].

Neurons of the upper cortical layers II-IV are generated between E13.5 and E16.5[68]. During neocortical development, Cux1 is expressed in both VZ and SZ, and functions as a transcriptional repressor of several genes involved in cell proliferation and differentiation. In addition, Cux1 regulates the dendritic arborization of pyramidal neurons[27,68,118]. In deep layer IV, we found that Cux1 is expressed at E14.5 and other studies have shown that these Cux1-positive neurons do not contain GABA, indicating that they represent glutamatergic neurons in layer IV[68,119]. This is followed by a peak of Cux1 expression in the mPFC at E16 and E18, where a large number of dendritic neurons are involved in the development of cortical circuits as well as in circuit refinement[118]. The expression of Satb2 is confined to the cerebral cortex with strict lateral and medial boundaries[23,25,120,121]. In the present study, Satb2 was initially detected at E14.5 in the neocortical SVZ and later in development in the upper layers of the PFC as well as in IZ[122] and in S1[25,122], followed by increased IL and PL expression at E16.5 and E18.5 in the mPFC subareas. At E18.5, most cells expressing Satb2 were predominantly found to occupy the upper cortical plate, a pattern that has also been identified by others[25]. In mutant Satb2$^{-/-}$ mice, the neurons of cortical layers II/III assimilated the fate of the cells of layer V[25,28,120], and upper- and lower-layer cells failed to form a correct corpus callosum and reach their sub-cerebral targets, respectively[25,122,123].

Together, the data presented show the existence of a balance in the actions of extrinsic and intrinsic factors to determine the cellular phenotypes during mPFC and S1 development. We further report a difference between the development of the dorsal areas, represented by the S1, and the PFC subareas. Our data demonstrate a delay in the proliferation, migration and maturation of the PFC when compared to the S1, presumably related to a higher neuronal complexity and functioning of the prefrontal region of the brain.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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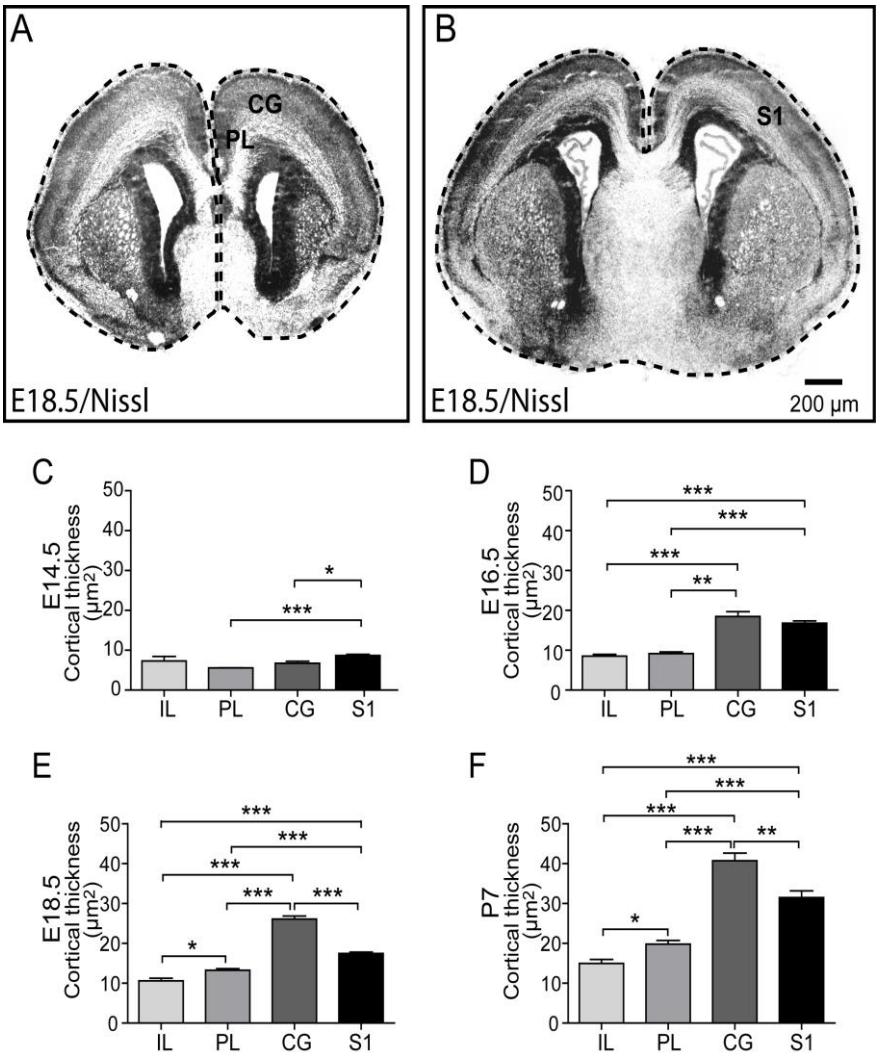


***Fig. 1. Cortical thickness of mPFC vs S1. (*A-B)** Nissl staining at E18.5 in mPFC and S1 coronal slices, respectively. **(C)** Total swatch area at E14.5 showing increased cortical thickness in S1 in comparison with PL and CG. **(D)** At E16.5, a significant increase in cortical thickness was found in S1 when compared with IL and PL areas. **(E)** At E18.5, a significant increase in CG area was found in comparison with IL, PL and S1, and in S1 in comparison with IL and PL. **(F)** At P7, the pattern of cortical thickness was similar to that at E18.5. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

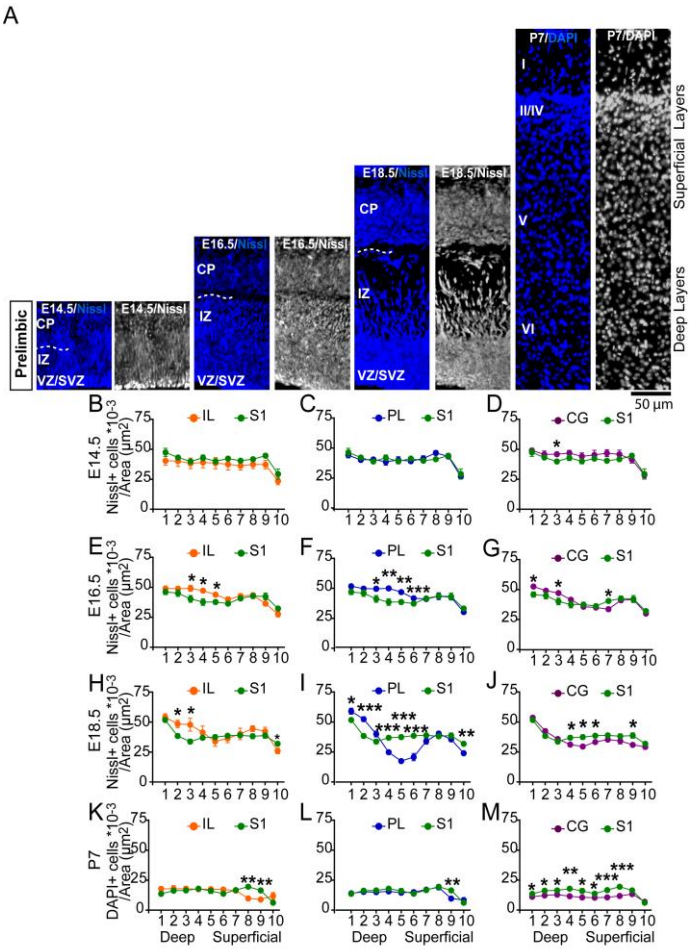


Fig. 2. Developmental timeline of radial expansion of cortex. **(A)** Cryosections showing total number of cells labeled by Nissl (E14.5, E16.5 and E18.5) and DAPI at P7. **(B-D)** Quantification of total number of cells per area at E14.5 between the cortical areas showing no significant differences, except by one deep domain in CG when compared with S1. **(E-G)** Significantly higher number of cells in deep and superficial layers in mPFC subareas when compared with S1. **(H-J)** Quantification of total number of cells at E18.5 showing differences between mPFC and S1. **(K-M)** Significantly higher number of cells in superficial layers in S1 when compared with IL and PL, and also in deep layers when compared with CG. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

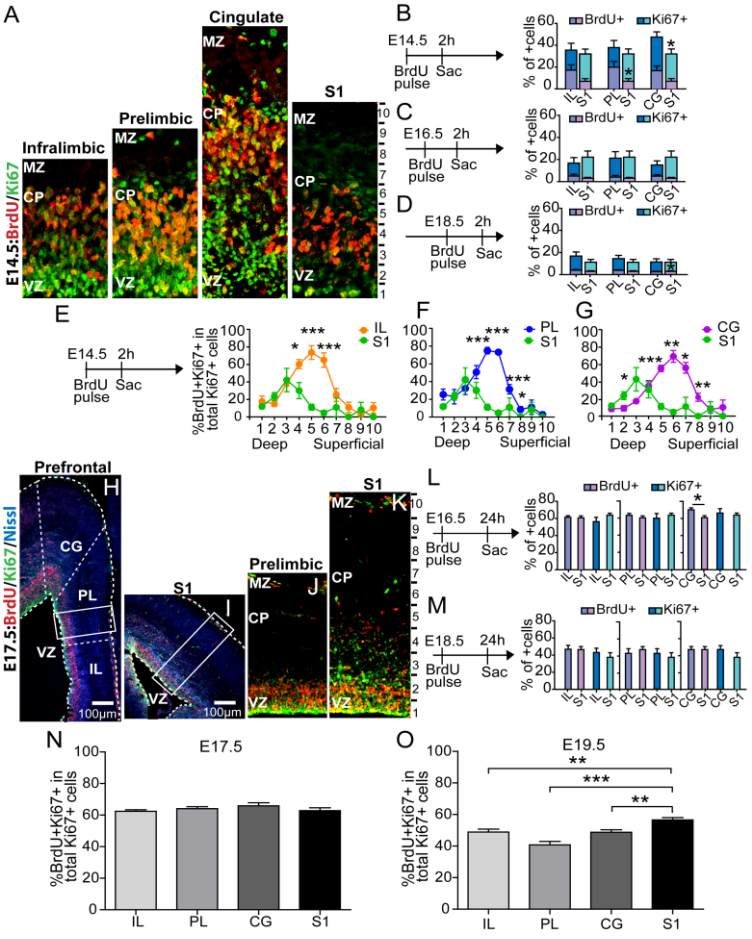


Fig. 3. Proliferation and migration of cells in mPFC vs S1 during corticogenesis. **(A)** Immunostaining showing cells in proliferation ($BrdU^+$ and $Ki67^+$ cells) after 2h of BrdU exposure. **(B-D)** Quantification of $BrdU^+$ and $Ki67^+$ cells, after 2h BrdU exposure, at E14.5 **(B)**, E16.6 **(C)**, E18.5 **(D)** and the cell cycle length showing a significantly higher percentage of proliferative cells in mPFC subdomains into deep and superficial layers at E14.5 when compared with S1. **(E-G)**. **(H-I)** Immunostaining showing $BrdU^+$ and $Ki67^+$ cells after 24h exposure to BrdU and in high magnification in **J-K**. **(L, M)** Quantification of $BrdU^+$ and $Ki67^+$ cells at E17.5 and E19.5 after 24h exposure to BrdU. **(N-O)** Quantification of the percentage of re-entry into the cell cycle after 24h exposure to BrdU ($BrdU^+/Ki67^+$). Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 3-5).

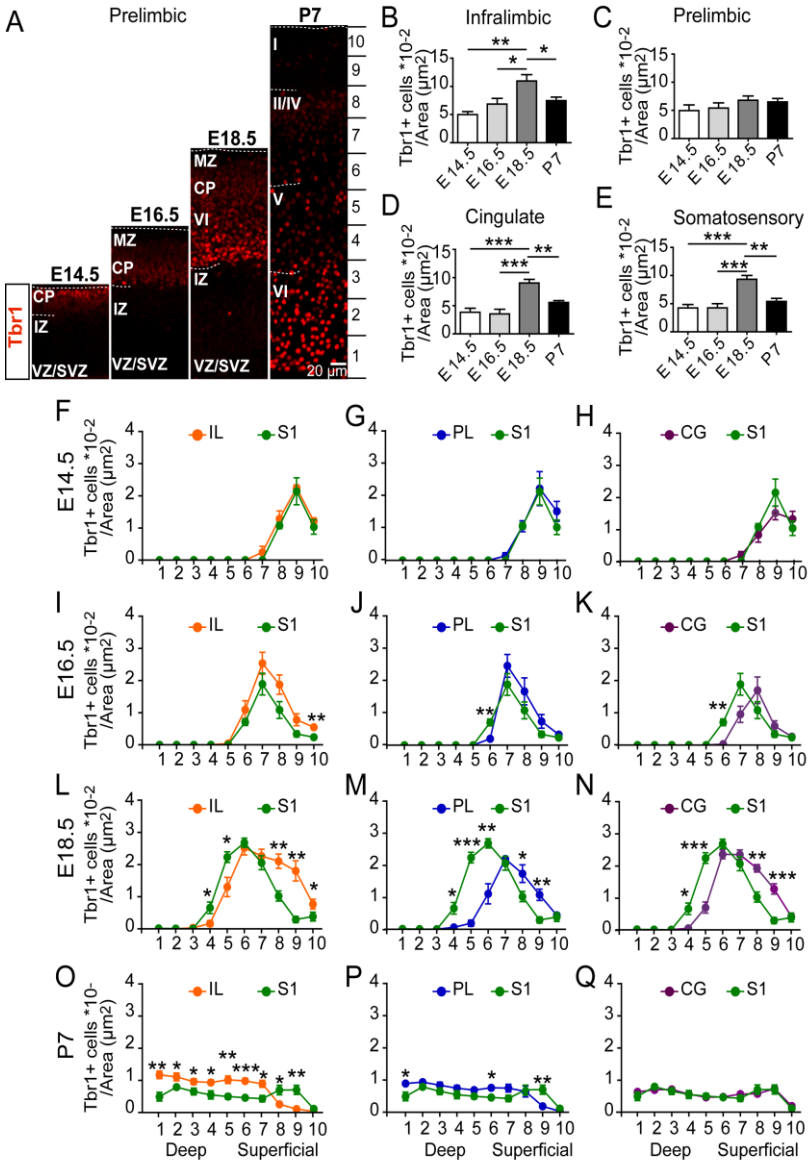


Fig. 4. Tbr1 is expressed by projection neurons in layer VI. **(A)** Immunostaining for Tbr1 at different time-points in PL cortices. **(B-E)** Quantification of total number of $Tbr1^+$ cells per area in IL, PL, CG and S1 at different time-points. **(F-Q)** Quantification of $Tbr1^+$ cells per area comparing mPFC subdomains and S1 in deep and superficial layers during development. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

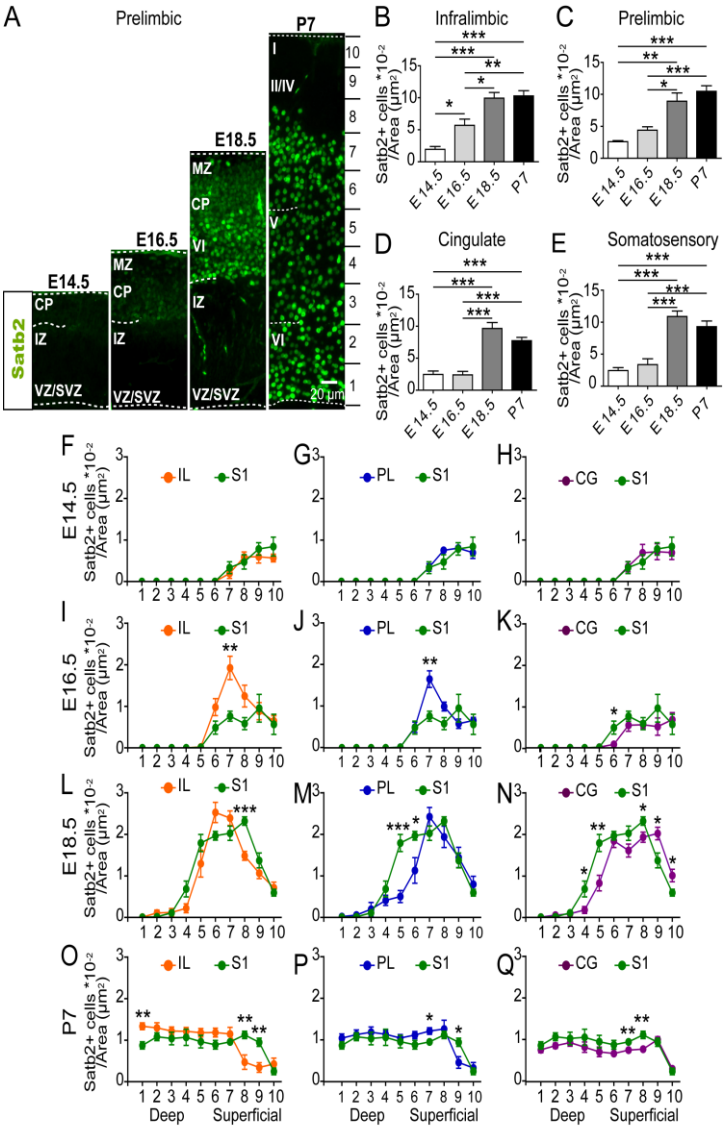


Fig. 5. Parallel developmental expression of Satb2 in mPFC and S1: **(A)** Immunostaining showing Satb2 labeling in PL slices at different time-points during development. **(B-E)** Quantification of Satb2$^+$ cells between mPFC and S1 at E14, E16, E18 and P7. **(F-Q)** Comparison of Satb2$^+$ cells per area in deep and superficial cortical layers of mPFC and S1. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

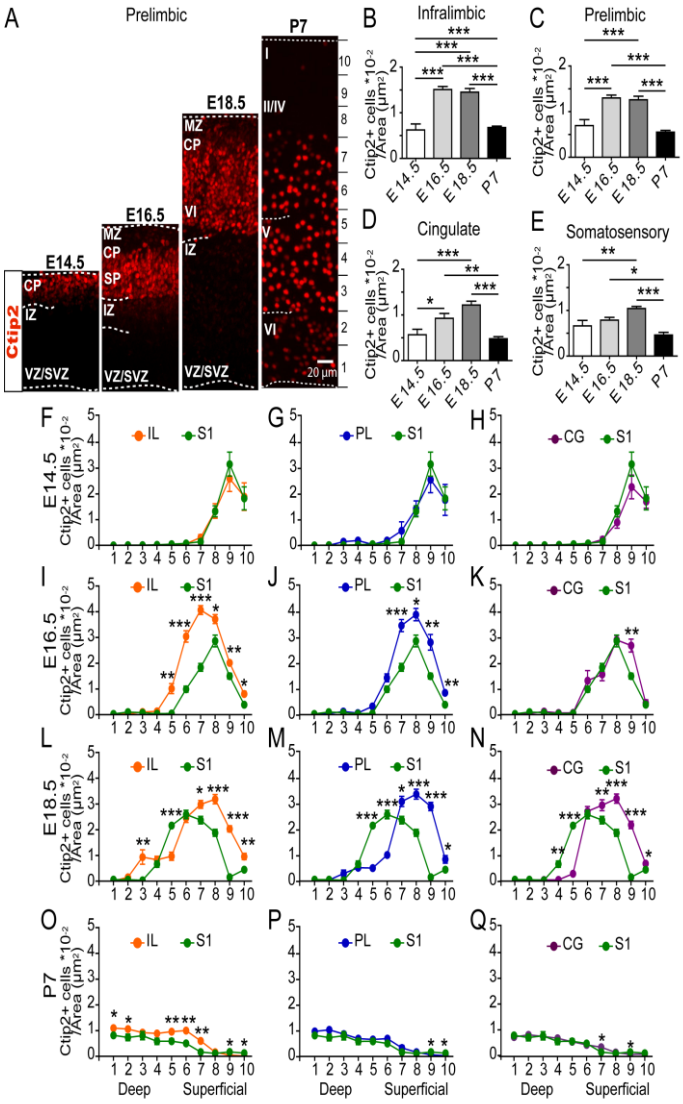


Fig. 6. Patterned expression of Ctip2 in neocortical Layers II–V. **(A)** Immunostaining showing the pattern of Ctip2 expression in PL slices during cortical development. **(B-E)** Quantification of Ctip2$^+$ cells per area in IL, PL, CG and S1 at different time points during development. **(F-Q)** Quantification of Ctip2$^+$ cells in deep and superficial layers of mPFC and S1 during development. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

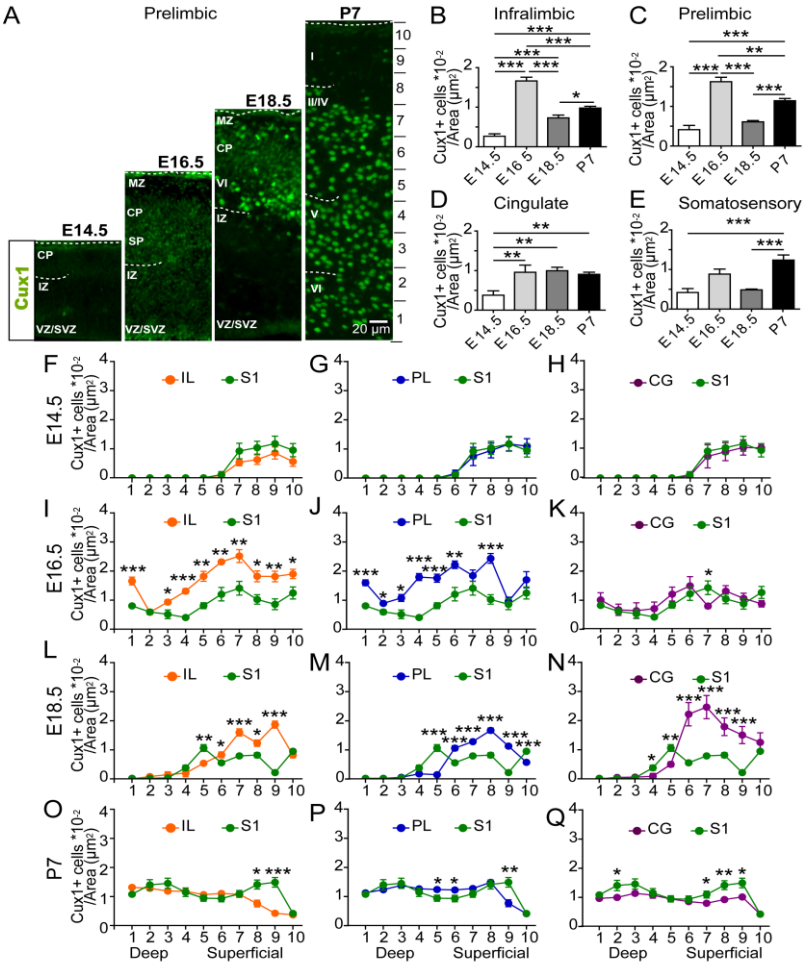


Fig. 7. Expression of Cux1 in late-born cortical neurons. **(A)** Immunostainig showing Cux1 expression during PL cortex development. **(B-E)** Quantification of $Cux1^+$ cell per area at different time-points comparing frontal with dorsal areas. **(F-Q)** Qualification of $Cux1^+$ cells in deep and superficial layers of mPFC subdomains compared with S1. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

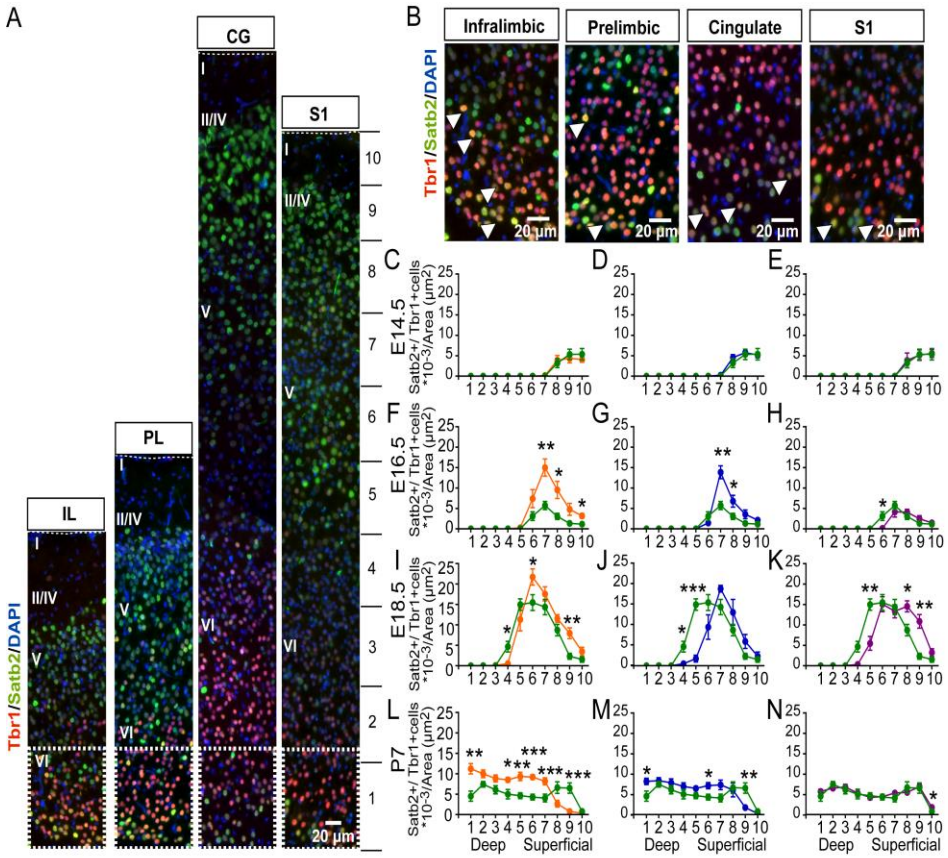


Fig. 8. Superficial domain vs deep domain: double labelling of Tbr1 and Satb2. **(A)** Immunostaining in mPFC and S1 at P7 showing $Tbr1^{+}/Satb2^{+}$ cells, and in high magnification in **(B)**. **(C-N)** Quantification of double-labeled cells in deep and superficial layers of mPFC and S1 at different time points during development. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

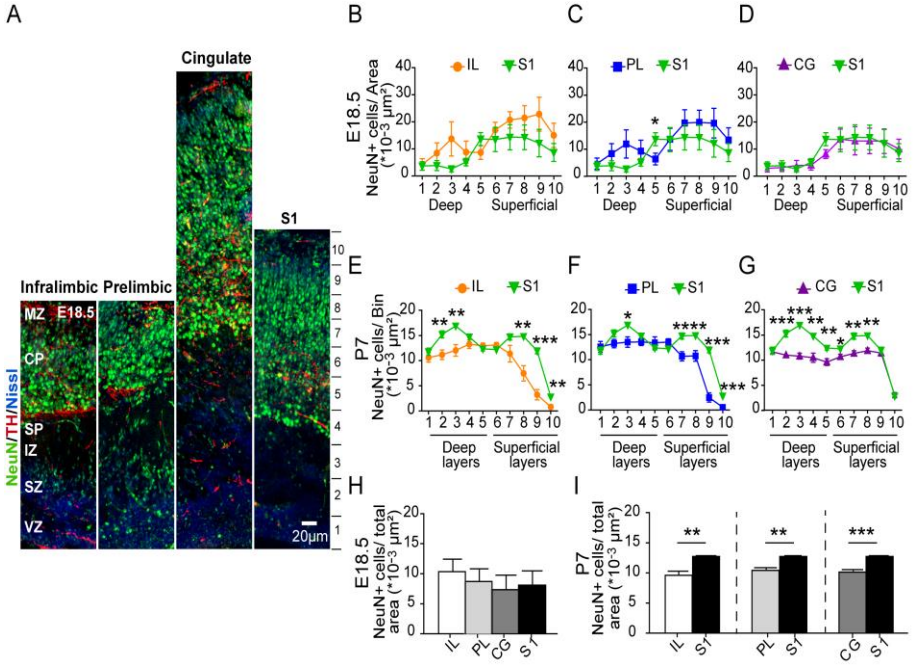


Fig. 9. Cortical maturation differences between frontal and dorsal areas. **(A)** Immunohistochemistry showing mature neurons ($NeuN^+$) and dopaminergic innervation (TH^+) in mPFC subdomains and S1 at E18.5. Quantification of $NeuN^+$ cells per area in deep and superficial layers of mPFC and S1 at E18.5 **(B-D)** and at P7 **(E-G)**. **(H, I)** Quantification of $NeuN^+$ cells in total swatch area in mPFC and S1 at E18.5 **(H)** and P7 **(I)**. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5-7).

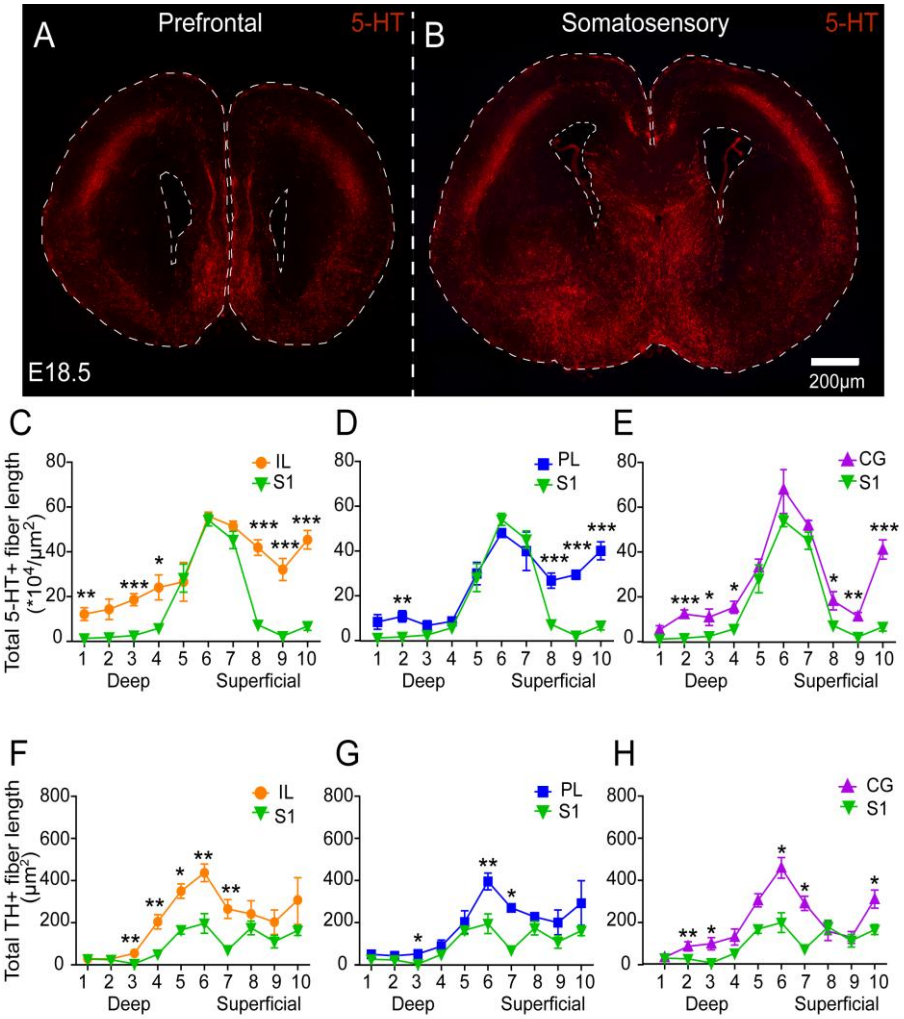
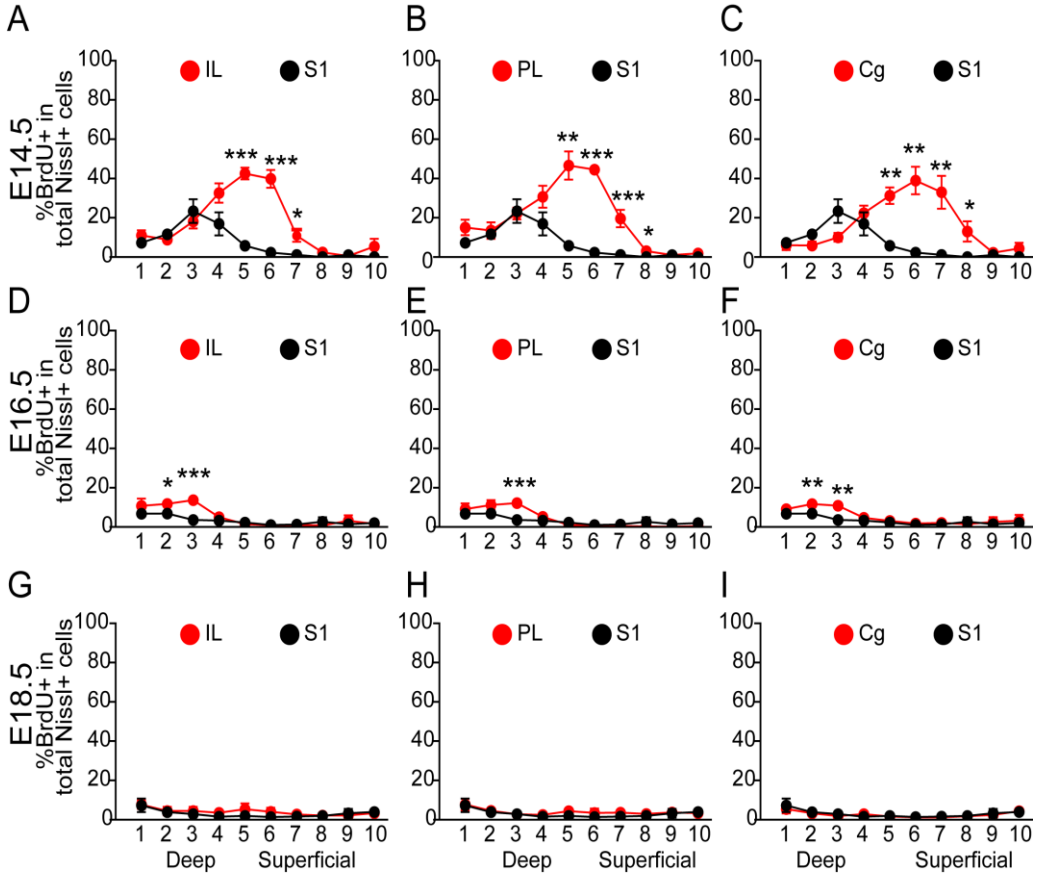
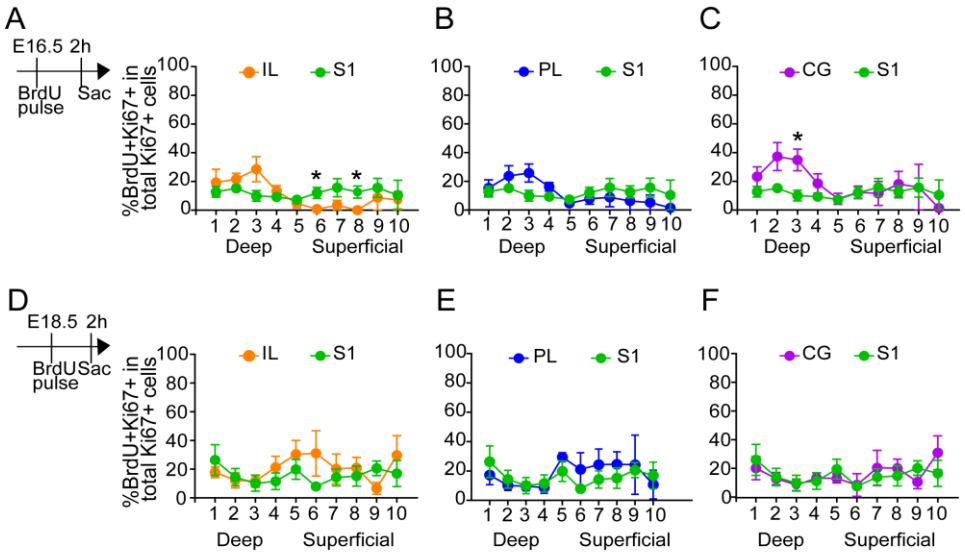


Fig. 10. The development of serotonergic and catecholaminergic innervation in the mPFC and S1. **(A, B)** Coronal slices of mPFC and S1 immunoassayed for 5-HT. **(C-E)** Quantification of 5-HT$^+$ fiber lengths per area at E18.5. **(F-H)** Quantification of dopaminergic innervation via TH$^+$ fiber lengths per area at E18.5. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5). 5-HT: serotonin, 5-hydroxytryptamine; TH: Tyrosine hydroxylase.



Supplemental Fig. 1. Proliferative activity of cells in mPFC subareas and S1 after 2-h exposure to BdrU. Quantification of the percentage of $BdrU^+$ cells at E14.5 **(A-C)**, at E16.5 **(D-F)** and at E18.5 **(G-I)**. Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5).



Supplemental Fig. 2. Cell-cycle re-entry of cells in mPFC subareas and S1. Quantification of cell re-entry into the cell cycle ($BrdU^+/Ki57^+$ cells) at E16.5 after 2-h exposure to BrdU **(A-C)**, at E18.5 after 2-h exposure to BrdU **(D-F).** Data show mean number of cells ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (n = 5).

3

Perturbed developmental serotonin signaling affects prefrontal catecholaminergic innervation and cortical integrity

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Abstract

Proper development of the medial prefrontal cortex (mPFC), crucial for correct cognitive functioning, requires projections from, among others, the serotonergic (5-HT) and catecholaminergic systems, but it is unclear how these systems influence each other during development. Here, we describe the parallel development of the 5-HT and catecholaminergic prefrontal projection systems in rat and demonstrate a close engagement of both systems in the proximity of Cajal-Retzius cells. We further show that in the absence of the 5-HT transporter (5-HTT), not only the developing 5-HT but also the catecholaminergic system, including their projections towards the mPFC, are affected. In addition, the layer identity of the mPFC neurons and reelin-positive interneuron number and integration are altered in the absence of the 5-HTT. Together, our data demonstrate a functional interplay between the developing mPFC 5-HT and catecholaminergic systems and call for a holistic approach in studying neurotransmitter systems-specific developmental consequences for adult behavior, to eventually allow the design of better treatment strategies for neuropsychiatric disorders.

Keywords: Neurodevelopment; Prefrontal Cortex (PFC); 5-HTT; TH; Cajal-Retzius

Introduction

Proper functioning of neural systems and correct targeting of their often-long projections to distant targets is crucial for cognitive performance. An important distant target of many neurotransmitters systems is the prefrontal cortex (PFC). The PFC is considered critical for executive and higher cognitive functioning[1-3]. Embryonic and early postnatal PFC development is directed by a sequence of intrinsic (e.g., proliferation, migration, and differentiation) and extrinsic (e.g., incoming projections/GABAergic interneurons) events which both can be affected in neurological and psychiatric disorders[1,4,5]. During development, the migration of newborn neurons establishes the characteristic inside-out layering of the PFC that furthermore receives numerous projections from various neurotransmitter systems, including the dopaminergic (DA), noradrenergic (NA), and serotonergic (5-hydroxytryptamine or 5-HT) systems[6-10]. It is unclear, however, how these systems interact during development and whether they influence each other. Cognitive and emotional disturbances are often attributed to the perturbed projection to the PFC of more than one neurotransmitter system[11-14], including the catecholaminergic and 5-HT systems, in neurological psychiatric disorders[15-22]. The 5-HT system clearly interacts with the catecholaminergic system in adulthood but it remains to be elucidated how they interrelate during PFC development.

The 5-HT system is one of the earliest to emerge and sends out projections (around E10.5 in mice, E12 in rat) to cortical areas during embryonic development[23,24]. The rostral raphe comprises 5-HT cell clusters in the dorsal raphe (DR, B6, and B7) and the median raphe (MnR, B5, and B8) that project to the forebrain with predominantly the medial part of the DR projecting to the PFC where they arrive around E16[25-27]. It has become increasingly clear that 5-HT, but also other neurotransmitters, can act as a neurodevelopmental signal instructing the brain as time proceeds[28-31]. In fact, 5- HT is able to modulate neurodevelopmental processes like proliferation, migration, and differentiation[30,32]. Within cortical areas, presumptive layer I Cajal-Retzius (CR) cells receive serotonergic and noradrenergic synaptic input during embryonic development and might therefore control their functioning[6,33,34]. Initially, CR cells secrete reelin which has been proven to be important for the specific inside-out patterning of cortical layers[35-37]. Later, CR cells develop into a heterogeneous population of GABAergic interneurons[38-40]. It remains to be determined however how exactly

disturbance of the developing 5-HT system influences the intrinsic neurodevelopmental events of the PFC.

The catecholaminergic system sends out projections to the forebrain approximately at the same time as the 5-HT system (E11.5 in mice, E13 in rat)[9,41-43]. Tyrosine hydroxylase- or TH-positive axons from the rostral part of the ventral tegmental area (VTA) arrive in the PFC around E15, somewhat earlier than the 5-HT system, in two streams within the subplate (SP) and the marginal zone (MZ) where the CR cells reside[9,44]. Although it remains speculative to what extent the catecholaminergic projections within the MZ are in synaptic contact with the CR cells, it is known that DA plays a developmental as well as a maturational role in prefrontal areas[45-49]. DAergic projections to the PFC are able to modulate proliferation, migration, and differentiation processes, and any interference during development could contribute to the cortical dysfunction in neuropsychiatric disorders[50]. Until recently, research was focused on understanding the ontogeny and functioning of separate neurotransmitter systems. However, comprehending the development and functioning of the brain in all its facets requires detailed knowledge of how various neural systems interact. In the adult brain, there is a clear interaction between the 5-HT and catecholaminergic projections towards the PFC, especially in the light of their engaged involvement in higher-order cognitive functioning[12,15,51]. Anatomically, the two systems considerably overlap in adulthood and seem to receive inputs from one another[52-54]. Less is known, however, about the extent to which the 5-HT and catecholaminergic systems influence each other during neurodevelopment[51-55].

Here, we describe the parallel development of the 5-HT and catecholaminergic systems in the rat between E16 and P6 with special emphasis on their common projection target, the medial PFC (mPFC). We show that in the absence of the 5-HT transporter (5-HTT), not only the 5-HT but also the catecholaminergic system, including TH-positive projections towards the mPFC, are affected. Within the mPFC, the reelin-containing CR cells are in close proximity to 5-HT and TH-positive fibers, and in the absence of the 5-HTT, they differ in number. We furthermore demonstrate that the identity of especially deep-layer neurons is altered in the 5-HTT$^{-/-}$ rats. Altogether, these data suggest that there is a functional interplay between the 5-HT and catecholaminergic systems during development leading to a distortion of the cytoarchitecture of the PFC. Thus, the possible interplay of multiple neural neurotransmitter systems during development has to be taken into account when studying the etiology of neuropsychiatric disorders.

Materials and Methods

Animals

The control neuroanatomical descriptions were performed on wildtype rats of the Wistar background purchased from a commercial breeder (Janvier, Labs, RjHan: WI; Hannover, Germany). The generation of the Slc6a4 wildtype (5-HTT$^{+/+}$) and mutant rats (5-HTT$^{-/-}$) has been described previously[56]. They were bred onto a Wistar genetic background. The day of the plug was considered to be embryonic day (E)0 and the day of birth to be postnatal day (P)0. All experiments were performed in compliance with the standard ethics guidelines of the European Community and in accordance with the recommendations of the local animal welfare committee (DEC) of the Radboud University. The protocol was approved by the DEC. Male and female embryos and pups were used indiscriminately in all experiments and sacrificed by decapitation.

Section preparation and immunohistochemistry

Brains were rapidly dissected from E16.5, E18.5, and E20.5 embryos, P6 pups, and P25 adolescents, and fixed by immersion for 0.5–1.5 h in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4. After fixation, brains were washed in PBS, cryoprotected in 30% sucrose overnight, frozen in M-1 embedding matrix (Shandon, Thermo Fisher Scientific Inc., Waltham, MA, USA) on dry ice in a plastic cup, and stored at − 80°C. Cryostat coronal or sagittal sections were cut at 16 µm, mounted as series of 6–8 on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored desiccated at −20°C. Cryosections were stained immunohistochemically and imaged as described previously[10,57] with the following exceptions; incubation of P6 and P25 sections with primary antibodies was done for either 3 h at room temperature (RT) or overnight (ON) at 4°C. Immunofluorescence was visualized using either an Invitrogen/Thermo Fisher Scientific EVOSTM FL Auto Imaging System with a high-sensitivity CMOS camera or EVOS FL Auto Software or using a Leica DMRA fluorescence microscope coupled with a DFC340FX digital camera and LASAF software. The primary antibodies, dilutions used, and antibody suppliers can be found in Table 1. The nomenclature to describe neurons and axons within different brain areas is as described previously by Schambra et al.[58] and Jacobowitz and Abott[59] and extended as outlined in[9,10] and in Supplemental Figure 1.

Data analysis

All data analyses were performed in a blinded fashion without knowledge of the animal's genotype. For assessing 5-HT- or TH-positive axon length and number of layer marker-positive neurons within the various subareas of the medial PFC (mPFC) of 5-HTT$^{+/+}$ and of 5-HTT$^{-/-}$ rats, three to five P6 pups were analyzed and two to four well-spaced (120 μm) sections at the same neuroanatomical level were imaged. A 0.1-mm-wide rectangle spanning the prefrontal wall was placed over the center of the subarea (either infralimbic, IL; prelimbic, PL; or cingulate cortex, Cg) of the mPFC. The overall cortical length of a subarea was divided into ten equal bins [bin 1 within the deep cortical layers and bin 10 within the presumptive layer I] within this rectangle, and 5-HT- or TH-positive axon length or number of layer marker-positive neurons were measured within each bin using ImageJ software including the NeuronJ plugin (NIH, Bethesda, USA). Data were normalized to a total length per bin, or to a percentage of the total number of cells and averaged for each pup. To better visualize and compare 5-HT and/or catecholaminergic innervation of wildtype and mutant mPFC, reconstructions of the individual fibers from two to three consecutive sections were obtained bilaterally using the NeuronJ plugin. Data were statistically analyzed by one-way ANOVA ($\alpha = 5\%$) using Graphpad Prism 6/Excel data analysis toolkit and expressed as means ± SEM. Surface area of TH^{+} (tyrosine hydroxylase, rate-limiting enzyme responsible for DA synthesis) area within the ventral midbrain was measured by dividing the area along the midline.

The surface area was then measured in μm^2 with Image J and averaged between left and right of two to three well-spaced sections. For each n, the same neuroanatomical level was chosen in terms of rostral-to-caudal extent. The measurements of 5-HT^{+} surface area of the B7 and B8 nuclei in the hindbrain was performed in a similar fashion with the exception that the entire nucleus was measured and not divided along the midline. 5-HT^{+} cell numbers of the B7 and B8 nuclei were counted in Adobe Photoshop in the same area used to measure the surface area in two to four well-spaced sections. Again, for each n, the same neuroanatomical level was chosen in terms of rostral-to-caudal extent. Data were statistically analyzed by one-way ANOVA ($\alpha = 5\%$) using Graphpad Prism 6/Excel data analysis toolkit and expressed as means ± SEM.

Results

Developing 5-HT and catecholaminergic systems targeting the mPFC

In rats, 5-HT neurons start extending axons by E12 and TH^+ axons by E13[9,23]. Both neurotransmitter systems send out ascending axonal projections to distant forebrain targets including the mPFC[9,23,43]. The ontogeny of both systems has been described in detail for each system individually, however little is known of the concurrent development of both systems. In order to examine the relationship between the developing 5-HT and catecholaminergic system, we immunostained cryosections of developing rat brains (E16, E18, E20, and P6) for TH and 5-HT. Sagittal sections suggest a close interrelationship of especially ascending TH^+ and 5-HT^+ axons (Fig. 1a–d). When we took a closer look at the origin of both ascending neural systems, the DA midbrain and the 5-HT DR and MnR at the coronal level, we were able to observe a close contiguity between the TH^+ and 5-HT^+ axons (Fig. 1e–t). Within the developing DR and MnR, besides the 5-HT neuron clusters, a combination of both TH^+ axons as well as TH^+ cell bodies could be observed (Fig. 1e–h). At E16, we observed overlapping positioning of the majority of the TH- and 5-HT- positive neurons (Fig. 1e). At E18, most of the TH^+ neurons and fibers could be observed dorsally within or near the B7 of the DR (Fig. 1e–h). Within the developing DA midbrain at E16, 5-HT axonal projections were closely intermingled with the developing and still migrating TH^+ neurons in both the substantia nigra (SN) and the VTA. The latter seemed to be more innervated by 5-HT projections throughout the course of development (Fig. 1i–l). TH^+ and 5-HT axons travel together towards forebrain targets and they run in parallel within the median forebrain bundle (MFB). We observed that TH^+ axons bundle in larger fascicles and reside more dorsal within the extent of the MFB when compared to the 5-HT axons (Fig. 1m–p). The 5-HT axons are located more ventral within the MFB and seemed to get more varicose as development proceeds (Fig. 1m–p). However, a large proportion of the TH^+ and 5-HT^+ axons coincide, suggesting a close contiguity within the MFB during development (Fig. 1m–p). Eventually, both neural systems reached numerous forebrain targets including the mPFC (Fig. 1q–t). Especially within the region of the lateral septum (ls), the catecholaminergic system concurred with the 5-HT system although there was only partial overlap. Within the mPFC, there are two main fascicle paths of TH^+ and 5-HT^+ axon bundles. At E18, the first path was observed as a robust bundle of fascicles and individual 5-HT-positive fibers were detected

above the subplate (SP) of all three mPFC subdomains (infralimbic, IL; prelimbic, PL; and Cingulate, Cg), although the fibers were less prominent within SP of the Cg (Fig. 1q–t). TH-positive fibers could also be observed at E18 but were less conspicuous and fasciculated (Fig. 1q–t). The other path of concurring TH^+ and 5-HT^+ fibers was found within the marginal zone (MZ) or the presumptive layer 1 where again the presence of 5-HT fibers exceeded that of the TH^+ ones (Fig. 1q–t). At E20, the innervation within the mPFC subdomains had increased in both paths and both systems innervated the cortical plate (CP) (Fig. 1s, t). In summary, there is a close intercalation and proximity of the catecholaminergic system and 5-HT system during development. They both innervate the mPFC in a similar pattern and time frame. Although the catecholaminergic system arrives earlier, the 5-HT system remains more prominent throughout development.

Close proximity of DA and 5-HT projections within the developing mPFC

There is a vast amount of information on the innervation of the mPFC by each of the catecholaminergic and 5-HT systems. However, less is known about the coinciding innervation of the mPFC during development. To address the coinciding localization and possible interaction of the catecholaminergic and the 5-HT system within the developing mPFC, we focused on the immunoreactive TH and 5-HT fibers within the different aspects of the mPFC subdomains. To show the proximity and intercalation of both neural systems, camera lucida drawings were obtained. At E16, no TH^+ or 5-HT^+ fibers could be observed within all cortical aspects of the individual subdomains (data not shown). At E18, the two above-mentioned paths could be observed especially in the IL and PL (shown) with TH^+ and 5-HT^+ varicose fibers running above the SP and within presumptive layer 1 (Fig. 2b, e, and h). Hardly, any innervation of the CP was present at this time point. At E20, both streams were still prominent with 5-HT^+ exceeding TH^+ fibers in the presumptive layer 1 and to a lesser extent above the SP (Fig. 2c, f, i, and k). Substantial innervation of the CP of mostly 5-HT^+ fibers could also be observed. At P6, extensive innervation by both neurotransmitter systems of all cortical aspects could be observed (Fig. 2d, g, j, and k). Yet, there was a clear 5-HT- and TH-positive band within layer I, which is most likely in close proximity of CR cells (Fig. 2b–j). TH^+ and 5-HT^+ axons seemed to run in close proximity especially early within development but, as was also clear from the camera lucida drawings, they appear to have their own distinct target

cells across layers. Thus, within the developing mPFC, both the catecholaminergic and the 5-HT systems are present and innervate cortical areas via the SP and MZ. Later, a large variety of neurons within the mPFC cortical layers get innervated by both the catecholaminergic as well as the 5-HT system.

The developing catecholaminergic system is affected in the absence of the 5-HTT

It is well accepted that 5-HT has an important neurodevelopmental role[30,60-62]. In previous work, we demonstrated that in the absence of 5-HTT, the amount of 5-HT-positive fibers increased dramatically within certain cortical layers of the IL and PL and to a lesser extent of the Cg ([10] and Fig. 3, Supplemental Figure 1). This raises the question of how elevated levels of extracellular 5-HT during development might influence other neural systems. To address whether the catecholaminergic system would be affected, we studied P6 brains of the 5-HTT mutant rat model. First, we tried to recapitulate our previous results: the 5-HT innervation of the subdomains of the mPFC was higher in 5-HTT$^{-/-}$ animals (n = 5) compared to wildtype (n = 5). Indeed, we again observed a significant increase of total 5-HT innervation in both the IL ($p = 0.000016$) and PL ($p = 0.000059$) and to a lesser extent in the Cg ($p = 0.11049$; Fig. 3c, f, i). Strikingly, we discovered that the catecholaminergic innervation of the mPFC subdomains is also affected in 5-HTT$^{-/-}$ animals. Within the IL and PL, we found a significant increase of TH^{+} innervation in especially the deeper layers and in bins 10 and 8 (more superficial layers) for the IL and PL of 5-HTT$^{-/-}$ animals, respectively (Fig. 3a, b, d, and e). Interestingly, the TH^{+} innervation of the CG tended to be higher in the deeper cortical layers whereas it was significantly lower in the more superficial layers, mimicking the results we obtained before for the 5-HT innervation[10] (Fig. 3g–h).

As the TH^{+} and 5-HT^{+} innervation of the mPFC was affected in 5-HTT$^{-/-}$ animals, the question remained whether also the source of the TH^{+} rostral VTA or rVTA) and 5-HT^{+} (DR and MnR) prefrontal fibers were affected by changes in 5-HT levels during development. To this end, we measured the surface area of the DA midbrain including the rVTA and the SN of both 5-HTT$^{-/-}$ P6 animals (n = 3) and their control counterparts (n = 3). The total amount of surface area comprising TH^{+} neurons was significantly lower in 5-HTT$^{-/-}$ animals as compared to controls ($p = 0.013$; Fig. 4g). In addition, there seemed to be more axons leaving the VTA area and TH-

positive neurons looked sparser and more disorganized, especially within the SN (Fig. 4d, e). We furthermore measured the total surface area of the raphe B7 and the B8 cluster in P6 5-HTT$^{-/-}$ animals and wildtypes, counted the 5-HT-positive cells in both clusters, and calculated the cell density. The total surface area comprising 5-HT neurons was significantly larger in both the B7 and the B8 cell cluster in 5-HTT$^{-/-}$ animals compared to controls ($p = 0.01$ and $p = 0.006$, respectively) Fig. 4n, q). This was reflected by an increase in length of the B7 cluster and an increase in width of the B8 cluster ($p = 0.031$) and ($p = 0.037$) respectively; Fig. 4o, r). Additionally, the total amount of 5-HT-positive cells within each cluster was significantly lower, resulting in a lower cell density for both cell clusters ($p = 0.002$ and $p = 0.0002$, respectively; Fig. 4m, p).

Together, we can conclude that when 5-HTT is absent during development, the mesoprefrontal catecholaminergic system and rhomben prefrontal 5-HT system are affected.

Reelin in relation to 5-HT and catecholaminergic signaling

The 5-HT fibers within the MZ have been shown to contact CR cells, and thereby control reelin release[6]. By combining immunostaining for 5-HT and for TH, we observed that there are numerous TH^{+} and 5-HT^{+} projections running through the MZ where the CR reside. The question remains, however, to what extent the absence of 5-HTT interferes with reelin release, either directly or indirectly, through altered 5-HT or other projections. To visualize the proximity of TH^{+} and 5-HT^{+} fibers with CR cells, we immunostained cryosections of E18 and E20 with either 5-HT or TH in combination with reelin. Throughout cortical regions, including all subdomains of the mPFC, 5-HT- and TH-positive fibers running through the MZ are in close proximity with CR cells (Fig. 5a–h). Confocal images showed that some varicosities were indeed contacting the CR cells (Fig. 5j and Supplemental Figure 2). Triple-labeling the cryosections with TH, 5-HT, and reelin revealed that the 5-HT and TH fibers both contact the CR cells, albeit at different sites (Fig. 5i), suggesting that besides the known 5-HT, also the TH fibers are in the vicinity to be able to influence CR cell output and possible reelin release.

Prefrontal cytoarchitecture is affected in the absence of the 5-HTT

The fact throughout development, 5-HT and TH fibers reside in close proximity with CR cells and that they are affected in the absence of 5-HTT,

made us speculate whether these innervation alterations could have impacted cortical build-up. To address the question to what extent layer-specific markers were affected, we immunostained mutant and wildtype P6 cryosections for a deep-layer marker, Tbr1 (Fig. 6a–c) and an upper-layer marker, Cux1 (Fig. 6d–f). Indeed, both markers were severely affected in their expression pattern (Fig. 6). The percentage of cells affected was significant in all ten bins but was most striking for the bins in deeper layers, specifically for Tbr1 (Fig. 6a–c). The total number of Cux1- and Tbr1-positive cells was significantly affected as well (Supplemental Figure 3). This is in coherence with our previous findings demonstrating that Satb2, a layer 2–5 marker was severely affected[10]. The total number of cells (DAPI-positive) was not significantly different in IL ($p = 0.2$), PL ($p = 0.9$), and CG ($p = 0.9$); (Supplemental Figure 3), suggesting that the altered layer marker expression is due to an altered identity. To investigate marker expression at a later developmental age, we immunostained mutant and wildtype P25 cryosections for two different deep-layer markers; Tbr1 and Satb2 (Fig. 6k–o). Expression levels of both markers were more restricted to the deeper layers in the wildtype brains and most affected when 5-HTT was absent (Fig. 6m–o). This suggests that, even though the percentage of cells expressing deep-layer markers catch up a bit at P25 reflecting some sort of developmental delay, a large portion will not be able to express these markers. Reelin-positive interneurons will dispense throughout the cerebral wall in early postnatal ages[36,37,63]. Even though the ontogeny of reelin-positive CR cells and the reelin-positive GABAergic interneurons differ in their ontogeny[64], we pursued to find out whether the number and integration of reelin-positive cells was affected by the lack of 5-HTT during the course of development. We counted the reelin-positive cells in the P6 mutant and the control animals. We discovered that in all subdomains of the mPFC, the number and distribution of reelin-positive neurons was affected in the 5-HTT$^{-/-}$ animals as compared to wildtypes (Fig. 7). In the IL, only bins 2, 5, and 6 showed lower number of reelin-positive cells (Fig. 7a, b), while in the PL, all bins except for 4, 5, and 8 showed significant lower numbers of reelin-positive cells (Fig. 7d, e). Within the CG, bins 4, 6, 9, and 10 showed significant lower numbers of reelin-positive cells (Fig. 7g, h). Together, we found a decrease in the total number of reelin- positive cells in all subdomains although the number did not reach significance within the IL (Fig. 7c, f, and i). This suggests that the integration and/or number of reelin-positive interneurons within the subdomains of the mPFC is affected as well.

In all, we observed a strong interdependence between the developing 5-HT and catecholaminergic system. In the absence of 5-HTT, we found significant differences in the shape (rVTA) and in the shape and content (DR/MnR) of the origins of both neural systems, a striking increase of both 5-HT and catecholaminergic innervations of the mPFC, altered deep-layer identity of mPFC neurons, and a decrease in reelin-positive cells, and which is summarized in Fig. 8.

Discussion

In this study, we show the coinciding development of the 5-HT and the catecholaminergic systems between E16 and P25 within their origin, their outgrowing projections through the MFB and their common projection target, the mPFC. In addition, our results demonstrate that in the absence of the 5-HTT and next to the 5-HT, also the catecholaminergic system and their projections towards the mPFC are altered. We further demonstrate that within the mPFC, the TH and 5-HT fibers are in close proximity to reelin-containing CR cells and are different in number when 5-HTT is lacking. We also observe that, with differences in 5-HT/TH innervation of the mPFC, prefrontal cell identity is altered. Altogether, these data suggest that there is a functional interplay between the 5-HT and catecholaminergic systems during development with an effect on the proper cytoarchitecture of the PFC.

Catecholaminergic and 5-HT control of prefrontal development

It is now well accepted that 5-HT plays an important role during neurodevelopment and that any disturbance of the system could add to the risk of developing neuropsychiatric conditions[30,65-67]. There is considerable genetic diversity among 5-HT neurons resulting in a vast and meticulously constructed network projecting to a large variety of targets[8,24,68,69]. The development of this heterogeneous pool of 5-HT neurons is under the control of intrinsic factors (e.g., transcription factors) and in interplay with extrinsic factors (e.g., guidance cues or cell adhesion molecules) that can steer the targeting projections[70-75]. Prefrontal 5-HT neurons arise in the rostral raphe cluster and the ascending axonal projections bundle up within the MFB and the fascicles traverse through the septal area towards the mPFC[6,23,76]. Here, they are bundled in two paths; one within the superficial MZ and one on top of the SP underneath the CP which they innervate after a short waiting period[6,23,77]. Essentially, the same developmental trajectory holds true for the

mesoprefrontal catecholaminergic projections, although the catecholaminergic system reaches the mPFC earlier[42,43,49]. Within the MZ and presumptive layer I of the mPFC, we observed 5-HT- and TH-positive varicosities in very close proximity of the reelin-positive CR cells. Synaptic structures, stained with either pre- or postsynaptic markers, could shed light on to what degree the CR cells receive TH- and/or 5-HT- synaptic inputs during development. In addition, varicosities do not necessarily have to imply synapses but can be a reflection of the complex that uses diffuse/volume transmission to communicate[78-80]. Nonetheless, there is certainly a spatial closeness of 5-HT^{+} as well as TH^{+} fibers that could imply the ability to influence CR output and maybe even reelin release. Reelin is known for its ability to direct cortical layer formation[81,82]. However, the developmental role of the effect of 5-HT, DA, or other neurotransmitters on reelin release needs to be further characterized.

Direct or indirect effects of 5-HTT on cortical integrity

One critical way by which extracellular 5-HT levels can be controlled is through expression of 5-HTT. The transporter can clear 5-HT from the synaptic cleft to maintain homeostasis[83]. Remarkably, the expression of 5-HTT is already quite robust in early development, even before serotonergic axons have reached their targets[60,84]. What does this imply? It is known that there is an extra-embryonic source of 5-HT from the placenta that could regulate certain aspects of central nervous system development[30,85,86]. Yet, many questions remain. Narboux-Nème and colleagues[60] elegantly showed a transient 5-HTT expression within layers II, V, and VI of the mPFC at E15.5, thus even before 5-HT fibers reach the mPFC. Could it be that these deep-layer cortical neurons are most affected by the absence of 5-HTT and the resulting elevation of extracellular 5-HT? We indeed observed that in both layers V and VI as well as more superficial aspects of the prefrontal subareas, 5-HT- and TH-innervation were altered in the absence of 5-HTT. In addition, in our experiments, the number of predominantly deep-layer but also of the superficial-layer neurons was affected in the absence of 5-HTT at P6. At P25, however, we still see this effect for two different deep-layer markers (Tbr1 and Satb2); however, there is some percentage expression that does express these markers in the mutant suggesting a developmental delay. There are no indications that proliferation or migration (data not shown) was affected. Altamura and colleagues showed that in 5-HTT-deficient mice there are differences in cell density and layer thickness[87]. Does this mean that there is

a different window of expression of layer-specific markers Cux1 and Tbr1 when 5-HTT is absent? At this point, it is hard to conclude whether this change in expression of layer markers is due to cell-autonomous effects (absence of 5-HTT) or cell non-autonomous effects (differences in innervation patterns/reelin effect), or a combination of both. Experiments using conditional mutants[88] that have a cell-type-specific deletion of 5-HTT could shed more light on this.

Interaction of the developing catecholaminergic and 5-HT systems

Numerous studies have proven that there is an interdependence between 5-HT and DA[11,15,16,18,89], although less is known about this phenomenon during development. Both systems are able to influence neurodevelopmental events such as proliferation, migration, and differentiation[30,51,62,90]. But can one system be facilitated by the other during development? We showed that the TH^+ projections within subdomains of the mPFC are altered in absence of the 5- HTT. Expression of 5-HTT has been found within virtually all DR neurons[60,84,91]. Are 5-HTT-deficient DR neurons able to alter the course of TH^+ projections? And if yes, what are the exact neurodevelopmental events of the developing DA neurons that can be influenced by the 5-HT system and at what developmental time points? Alongside the changes in 5-HT receptor expression[92,99], the expression of DA receptors and transporters might be altered as a consequence of a disrupted 5-HT system and DA system. This can result in changes in system sensitivity and excitability which would have extreme consequences for the maturation of cortical cells. For example, reelin-positive interneurons express the $5\text{-}HT_{3A}$ receptor[64,100]. Could changes in the 5-HT projection system have led to the changes we observed in the number and distribution of the reelin-positive cells? We need to have a complete picture of the spatial and temporal aspects of these developing systems in order to be able to design preventive measures or curative treatment without any side effects.

Guidance of interconnected systems

The 5-HT projections to target-selective forebrain regions are under the control of classical guidance molecules[2,70,75]. The 5-HT system is furthermore capable of modulating the responsiveness of axons to guidance cues such as netrins[62]. However, the catecholaminergic system reaches the mutual forebrain targets earlier than the 5-HT system does. Can it be that the

altered catecholaminergic system development due to the absence of 5-HTT influences the developing mPFC earlier than the 5-HT and in a different manner? Or is it the absence of 5-HTT in target areas and within guidepost positions along the way that has changed guidance cue expression or the responsiveness of the TH^+ fibers? Even though the DR and MnR project to different targets, their development involves a common guidance family[101]. The differential expression of EphA5 and ephrina5, and consequential difference in Eph ephrin signaling, steers the region-specific 5-HT innervation. Whether developing catecholaminergic projections within the same temporal and spatial window are also responsive to the same guidance cues as the 5-HT axons headed towards the mPFC remains to be established. Neurodevelopmental processes may diverge in different brain regions and at various developmental time points. For example, the ontogeny of neurotransmitter systems can be affected by risk factors and aberrant projections may result[1,9,10,22,50]. Depending on the type of risk factor involved and their sensitive windows, which control the timing of when a disorder becomes overt, it is either the mPFC itself or its connected brain areas that may maldevelop. Yet, the net effect of faulty projections on other developing systems remains to be determined. Longitudinal studies at the systems level, including a complete inventory of the expression of a variety of neurotransmitter receptors and transporters in relation to the developing projections traveling together and their actions within the mPFC are needed. This would generate an important wealth of knowledge in order to understand the complexity of these interacting systems during development.

Conclusions

Our study shows a functional interplay between the 5-HT and catecholaminergic systems during development. As expect- ed, due to the absence of the 5-HTT the 5-HT system was disturbed but we found that the catecholaminergic system was perturbed as well, together resulting in an altered maturation of the mPFC. Overall, the striking observation of both 5-HT and catecholaminergic hyperinnervation of prefrontal subregions highlights the need for precise system-oriented dissection of neural systems that concomitantly develop. The removal of only one building block may destabilize a plethora of interacting neurodevelopmental systems leading to impairment of cognitive functioning. This calls for more studies on the dissection of neurotransmitter systems-specific consequences on adult

behavior to eventually allow the design of better treatment strategies for neuropsychiatric disorders.

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Conflict of interest

The authors declare that they have no conflict of interest

Compliance with ethical standards

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Table 1 The primary antibodies, dilutions used, and antibody suppliers

Antibody	**Dilution**	**Company**
Rabbit anti-5-Hydroxytryptamine (5-HT)	1:1000	Sigma-Aldrich
Mouse anti-Satb2	1:500	Abcam
Mouse anti-Reelin	1:500	Chemicon
Mouse anti-Cux1	1:300	Abcam
Rabbit anti-Tyrosine Hydroxylase (TH)	1:1000	Millipore
Chicken anti-Tyrosine Hydroxylase (TH)	1:500	Abcam
Rabbit anti-Tbr1	1:500	Abcam

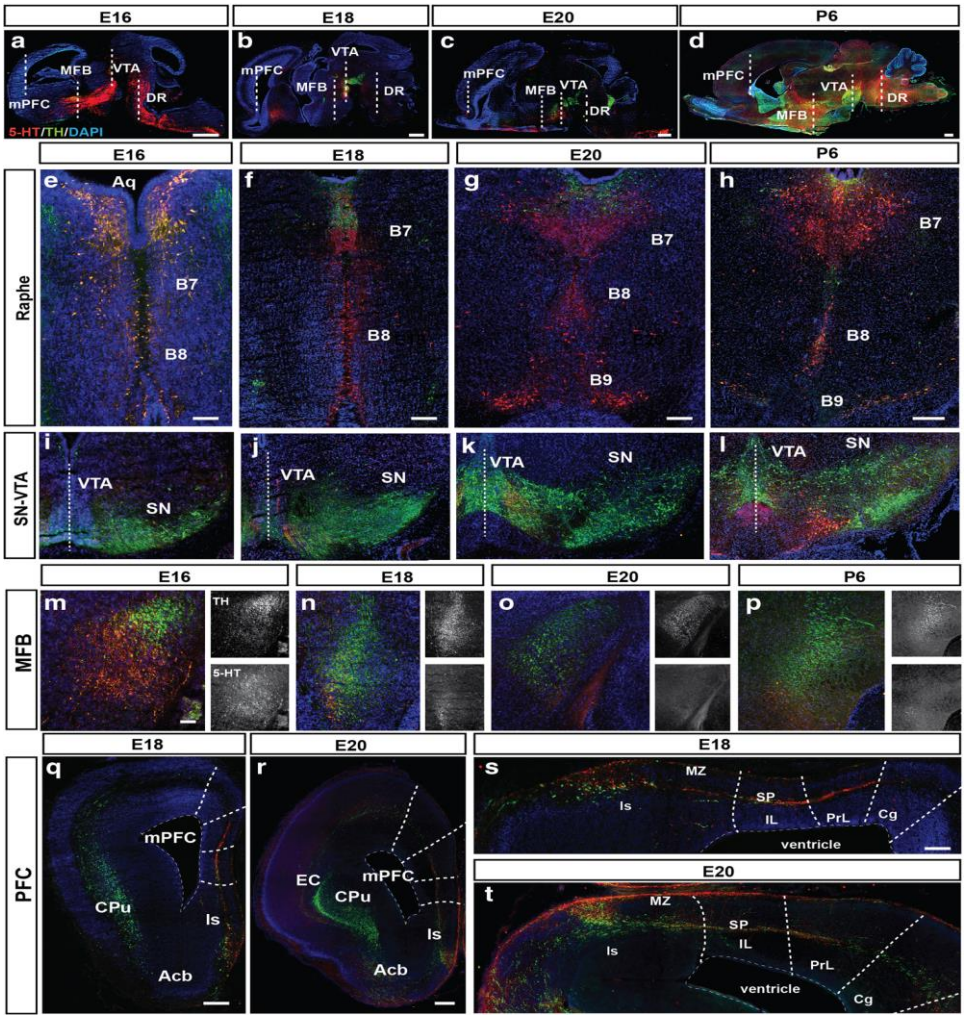


Fig. 1. Developing 5-HT and catecholaminergic systems target the mPFC. Sagittal cryosections of E16 **(a)**, E18 **(b)**, E20 **(c)**, and P6 **(d)** rat brains immunostained for 5-HT (red) and TH (green) and stained with DAPI (blue) to visualize cell nuclei showing the 5-HT and the catecholaminergic developing systems. Dotted lines indicate the coronal section levels. Enlargements of coronal cryosections of E16 **(e)**, E18 **(f)**, E20 **(g)**, and P6 **(h)** rat brains immunostained for 5-HT (red) and TH (green) and stained with DAPI (blue) showing the DR with the B7, B8, and/or B9 5-HT-positive cell clusters closely intermingled with TH-positive neurons and fibers. Enlargements of coronal cryosections of E16 **(i),** E18 **(j)**, E20 **(k)**, and P6 **(l)** rat brains immunostained for 5-HT (red) and TH (green) and stained with DAPI (blue) showing the rostral ventral tegmental area (rVTA) and the substantia nigra (SN) with the TH- positive cell clusters/fibers closely intermingled with 5-HT-positive neurons

and fibers. Dotted lines indicate the midline. Enlargements of coronal cryosections of E16 **(m)**, E18 **(n)**, E20 **(o)**, and P6 **(p)** rat brain immunostained for 5-HT (red) and TH (green) and stained with DAPI (blue) showing the medial forebrain bundle (MFB) with TH residing mainly in dorsal fascicles while 5-HT is present mostly within more caudal ones. Coronal (half shown) cryosections of E18 **(q)** and E20 **(r)** rat brains immunostained for 5-HT (red) and TH (green) and stained with DAPI (blue) showing the forebrain targets of both systems including the caudate putamen (CPu), the lateral septum (ls), the nucleus accumbens (Acb), and the medial prefrontal cortex (mPFC). Enlargements of the mPFC region of E18 **(s)** and E20 **(t)** rat brains showing a TH/5-HT- positive stream above the subplate (SP) and one in the marginal zone (MZ) in all three prefrontal subdomains (infralimbic, IL; prelimbic, PL; and cingulate, Cg). ls, lateral septum. Bar in a–d, 500 μm; e, 200 μm; f, 300 μm; g, 250 μm; h, 200 μm; i–l,300 μm; m–p, 200 μm; qandr, 300 μm; sandt, 250 μm.

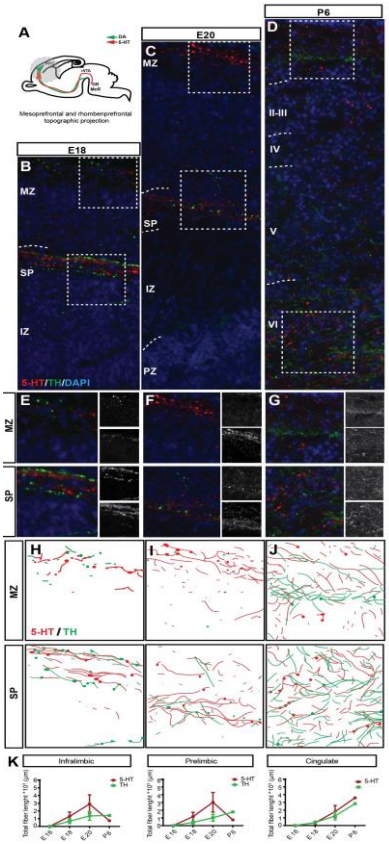


Fig. 2. Caecholaminergic and 5-HT interaction within the developing mPFC. **(a)** Schematic representation of a sagittal view of a developing embryonic rodent brain showing the catecholaminergic mesoprefrontal topographic projection (green arrow) and the 5-HTergic rhomben prefrontal topographic projection (red arrow) highlighting the forebrain target; the mPFC (within the gray circle). Enlargement of coronal cryosections of E18 **(b)**, E20 **(c)**, and P6 **(d)** rat brains immunostained for 5-HT (red) and TH (green) and stained with DAPI (blue) showing a cortical swatch of the mPFC IL subarea. Enlargements of the boxed areas in **B-D** showing TH- and 5-HT-positive fibers within the marginal zone (MZ, upper box) and subplate (SP, lower box) region of the E18 **(e)**, E20 **(f)**, and P6 **(g)** mPFC flanked by the gray-valued separated channels (TH upper and 5-HT lower box). (**h–j)** Camera lucida drawings of the boxed areas in **b–d** showing the proximity of the TH- (green) with the 5-HT- positive (red) fibers. (**k)** Quantification of the total length of TH (green) and 5-HT-positive (red) fibers within the three subareas (IL, PL, and Cg) of the mPFC. IZ intermediate zone, PZ proliferative zone. Bar in a, 100 μm; b, 80 μm; c, 70 μm.

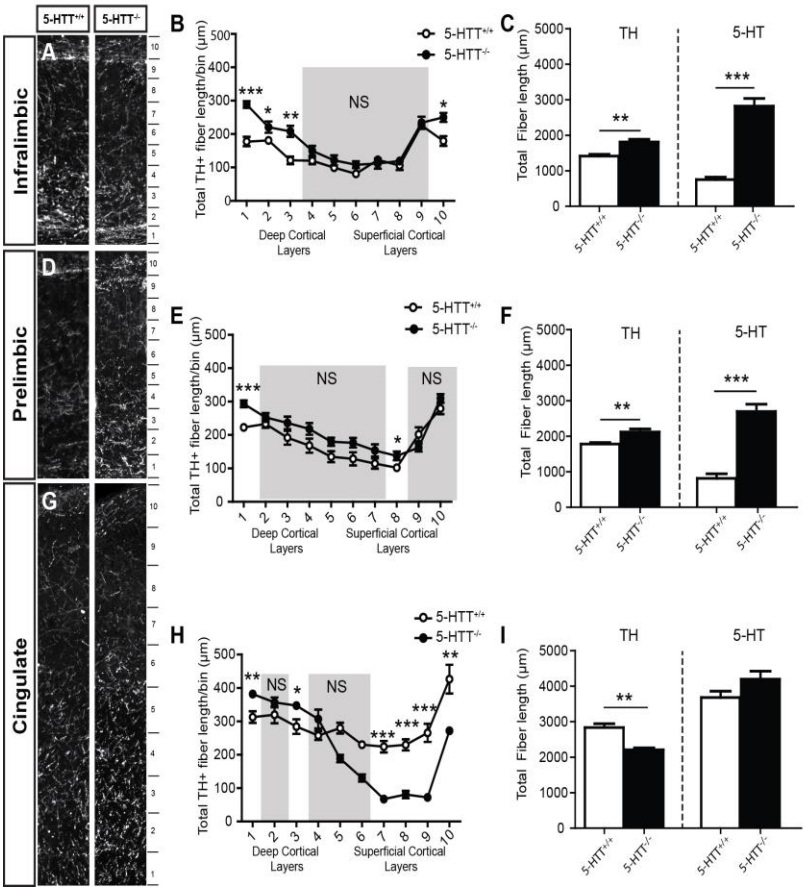


Fig. 3. Catecholaminergic innervation of the mPFC affected in 5-HTT-deficient rat model. Enlargements of cryosections of P6 5-HTT$^{+/+}$ and 5- HTT$^{-/-}$ rat brains showing prefrontal swatches of the IL **(a),** PL **(d)**, and the Cg **(g)** immunostained for TH (white). Quantification of the TH^{+} fiber length (in µm) within the bins indicated in a, d, and g in the IL **(b)**, PL **(e)**, and Cg **(h)** of 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ pups confirming the qualitative observations. The gray boxes represent the non-significant (NS) bins. Graphs in **b, e**, and h show average total length of TH-positive fibers per bin ± SEM. One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001. Quantification of the total TH-positive (left) as compared to the total 5-HT-positive fiber length (in µm) over the complete length of the prefrontal swatch in the IL **(c)**, PL **(f)**, and Cg **(i)** of 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ pups. Graphs in **c, f,** and i show average total length of TH- and 5-HT-positive fibers ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA. Bar in all, 50 µm.

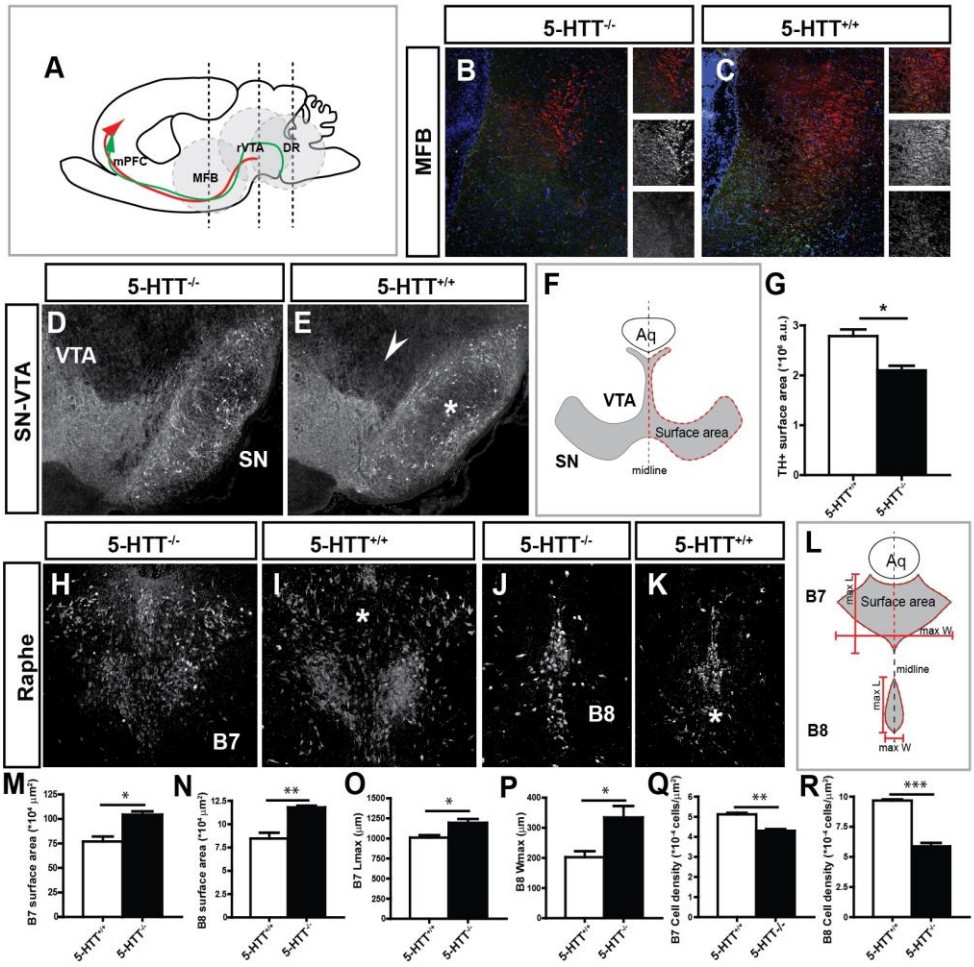


Fig 4. Catecholaminergic system is affected when 5-HT levels are perturbed during development. **(a)** Schematic representation of a sagittal view of a developing postnatal rodent brain showing the catecholaminergic mesoprefrontal topographic projection (red arrow) and the 5-HTergic rhomben prefrontal topographic projection (green arrow) towards the mPFC highlighting the DA origin rVTA, the 5-HT origin DR, and the MFB (within the gray circles). Enlargements of coronal cryosections of P6 rat brain immunostained for 5-HT (red) and TH (green) and stained with DAPI (blue) showing the MFB of 5-HTT$^{-/-}$ **(c)** compared to 5-HTT$^{+/+}$ animals **(b)** showing a higher level of defasciculation of TH^{+} fibers and a lower level of 5-HT fibers in the 5-HTT$^{-/-}$ pups. Boxed area shows the individual fascicles of the catecholaminergic (middle box) and 5-HT (lower box) system. Enlargements of coronal cryosections of P6 rat brain immunostained for TH (white) showing the rVTA of 5-HTT$^{-/-}$ **(e)** compared to 5-HTT$^{+/+}$ **(d)** animals showing more catecholaminergic fibers exiting

the VTA area (arrowhead) and fewer and less organized TH+ neurons in the SN (asterisk) of 5-HTT$^{-/-}$ pups. **(f)** Schematic representation of a coronal view of a developing DA midbrain including the VTA and SN showing the measured surface area (surrounded by red dotted lines). Aq aqueduct. **(g)** Graph showing the surface area occupied by TH+ fibers/neurons ± SEM which is significantly smaller for 5-HTT$^{-/-}$ pups. One-way ANOVA, *$p < 0.05$. Enlargements of coronal cryosections of P6 rat brain immunostained for 5-HT (white) showing the DR B7 **(h, i)** and B8 **(j, k)** cell cluster of 5-HTT$^{-/-}$ **(i, k)** compared to 5-HTT$^{+/+}$ **(h, j)** animals showing irregularities in both cell clusters (asterisks) of 5- HTT$^{-/-}$ pups. **(l)** Schematic representation of a coronal view of the developing 5-HT raphe area including the B7 and B8 cell clusters showing the measured surface area (surrounded by red dotted lines) and the maximal width (max W) and the maximal lengths (max L) of both clusters (red lines). Aq aqueduct. Quantification of the number of the 5- HT-positive neurons in the B7 **(m)** and B8 **(p)** cluster, the measured surface area of the B7 **(n)** and the B8 **(q)** area, the maximal length of the B7 cluster **(o)**, and the maximal width of the B8 cluster **(r)**. Bar in b and c, 200 μm; d and e, 300 μm; h–k, 100 μm.

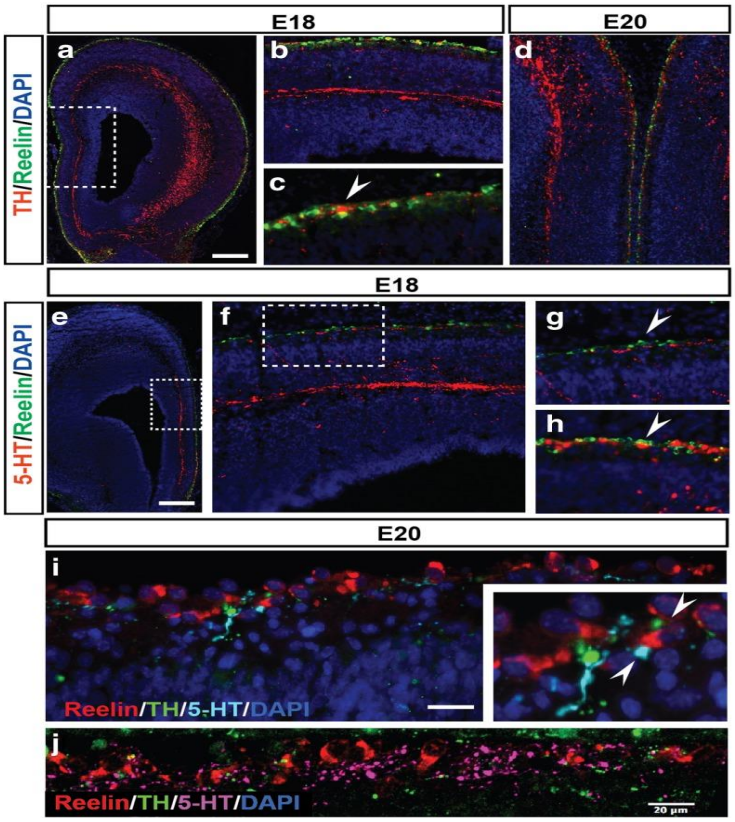


Fig. 5. The 5-HT and catecholaminergic systems are in close proximity of the CR cells. Coronal cryosections of E18 (**a–c, e**, and **f**) and E20 (**d, i**) rat brains immunostained for TH (red, **a–d**) or 5-HT (red, **e–h**) and reelin (green), counterstained with DAPI (blue). **b, c** Enlargements of the boxed area in a showing the TH fibers in close proximity to the reelin-positive CR cells. **d** Overview of the relation of TH innervation within the mPFC at E20. Coronal cryosections of an E18 rat brain immunostained for 5-HT (red, **e–h, i**) and reelin (green), counterstained with DAPI (blue). **f** Enlargement of the boxed area in e showing the 5-HT fibers in close proximity to the reelin-positive Cajal-Retzius (CR) cells. **g** Enlargement of the boxed area in f showing the 5-HT fibers in close proximity to the reelin-positive CR cells. h Enlargement of the septal region in e showing the 5-HT fibers in close proximity to the reelin-positive CR cells. **i** E20 cryosection stained for TH (green), 5-HT (light blue) and reelin (red) and counterstained with DAPI (blue). Inset shows the close proximity of the TH and 5-HT fibers with the CR cells. **j** Confocal image of the marginal zone showing TH (green) and 5-HT fibers (purple) in close proximity to reelin-positive CR cells (red). Bar in a and e, 300 µm; i, 30 µm; j, 20 µm.

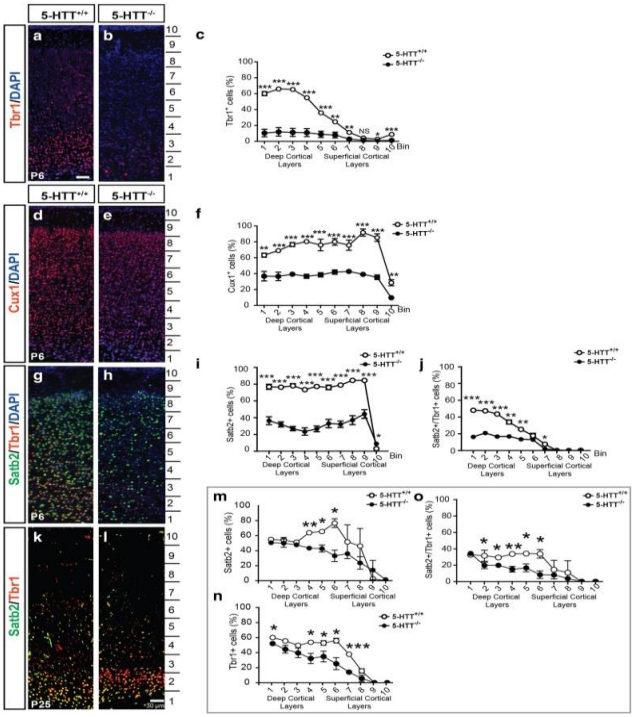


Fig. 6. Prefrontal cytoarchitecture is affected in the absence of 5-HTT. Enlargements of cryosections of P6 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rat brains showing prefrontal swatches of the PL immunostained for Tbr1 (red, **a, b)** or Cux1 (red, **d, e**) and counterstained with DAPI (blue). **b, e** Quantification of the percentage of Tbr1-positive **(c)** or Cux1-positive **(f)** neurons within the bins indicated in **a, b, d,** and **e. g, h** Enlargements of cryosections of P6 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rat brains showing prefrontal swatches of the PL double-immunostained for Tbr1 (red) and Satb2 (green) and counterstained with DAPI (blue). **i** Quantification of the percentage of Satb2-positive neurons within the bins indicated in **h. j** Quantification of the percentage of Satb2/Tbr1 double-positive neurons within the bins indicated in **h. k, l** Enlargements of cryosections of P25 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rat brains showing prefrontal swatches of the PL double-immunostained for Tbr1 (red) and Satb2 (green). **m** Quantification of the percentage of Satb2-positive neurons within the bins indicated in **l**. **n** Quantification of the percentage of Satb2-positive neurons within the bins indicated in **l. o** Quantification of the percentage of Satb2/Tbr1 double-positive neurons within the bins indicated in **l**. Graphs in c–o show average percentage ± SEM. One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001. Bar in a–l, 100 μm.

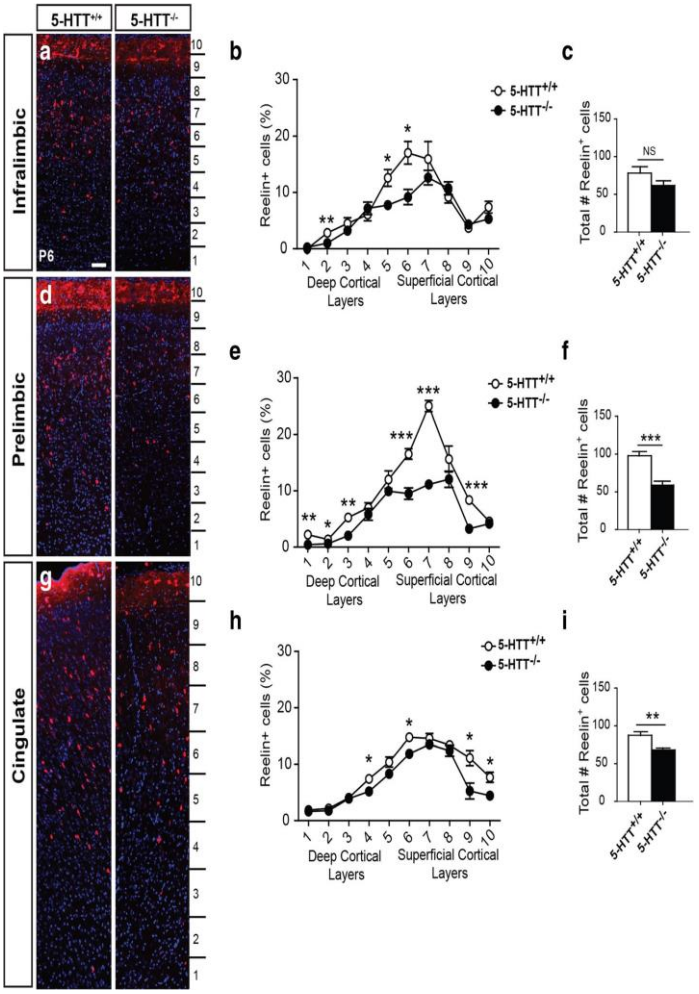


Fig. 7. The number of reelin-positive cells is diminished in the absence of 5-HTT. Enlargements of cryosections of P6 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rat brains showing prefrontal swatches of the IL **(a)**, PL **(d)**, and the Cg **(g)** immunostained for reelin (red) and counterstained with DAPI (blue). Quantification of the percentage of reelin-positive neurons within the bins indicated in **a, d,** and **g** in the IL **(b)**, PL **(e)**, and Cg **(h)** of 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ pups confirming the qualitative observations. Graphs in **b, e**, and **h** show average percentage of reelin-positive neurons normalized to total number of cells per bin ± SEM. One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001. Quantification of the total number of reelin-positive cells over the complete length of the prefrontal swatch in the IL (**c**), PL **(f)**, and Cg **(i)** of 5-HTT$^{-/-}$ (black bar) compared to 5-HTT$^{+/+}$ pups (white bar). Graphs in **c, f,** and **i** show average number ± SEM. One-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001. Bar in a–g, 100 μm.

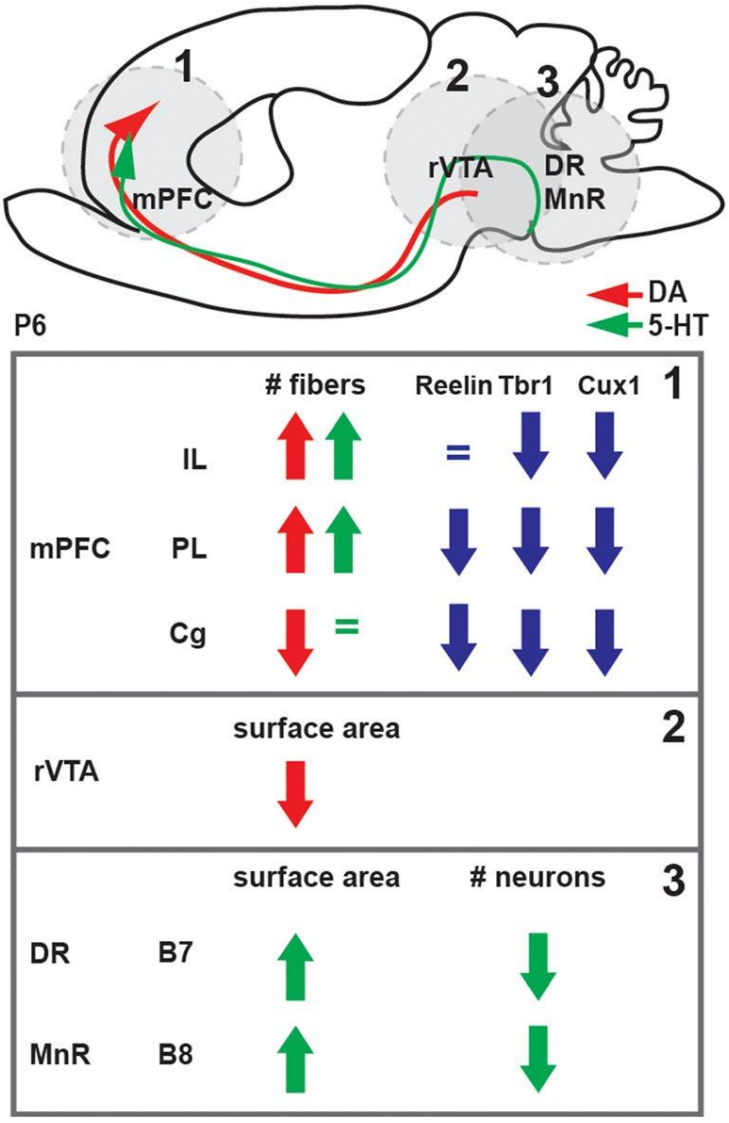
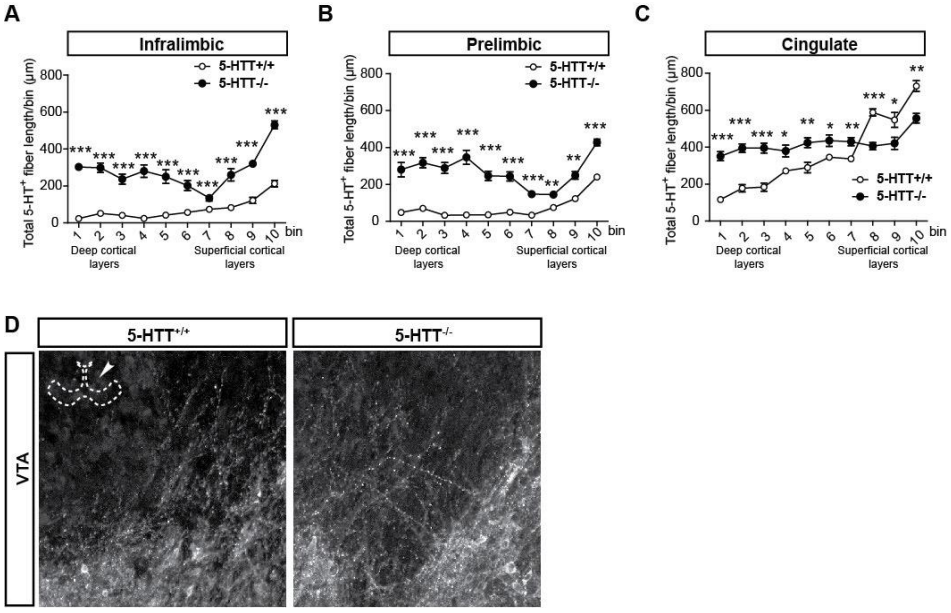
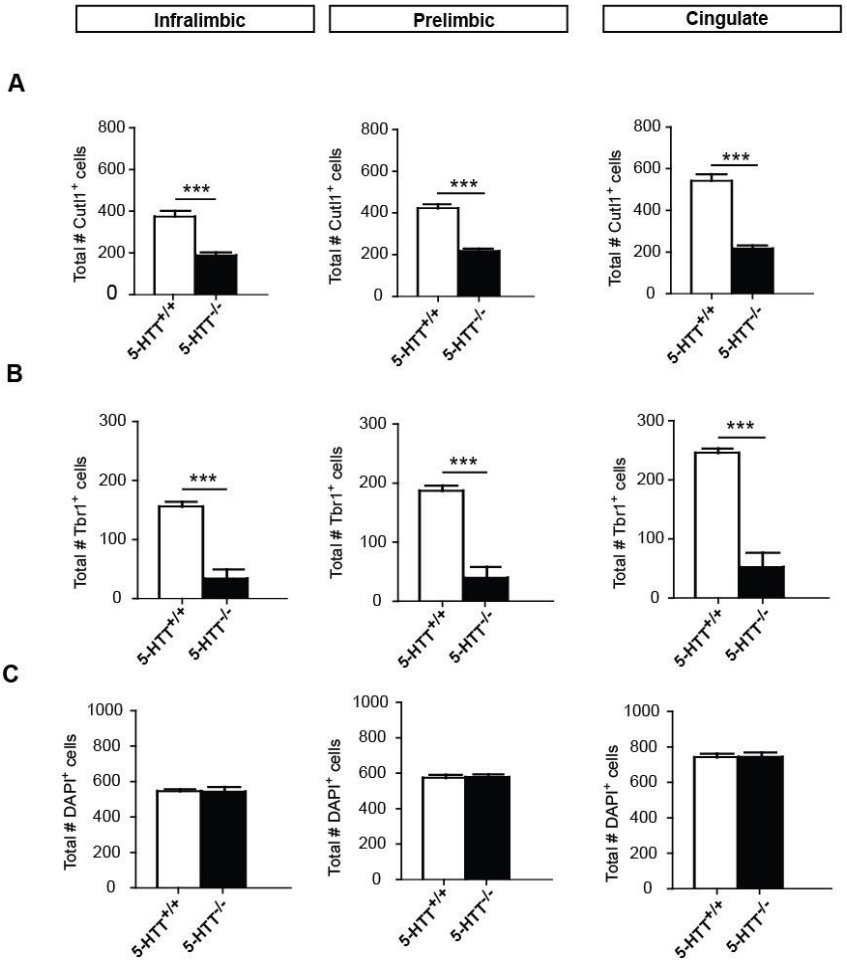


Fig. 8. Schematic overview of the results observed in the 5-HTT rat model. DA dopaminergic, Cg cingulate, DR dorsal raphe, 5-HT serotonergic, IL infralimbic, MnR medial raphe; mPFC medial prefrontal cortex, PL prelimbic, rVTA rostral ventral tegmental area.



Supplemental Fig. 1. 5-HT and DA systems affected in 5-HTT mutant rat model. (**A, B, C**) Quantification of the 5-HT fibers length (in µm) per bin in mPFC subareas: IL (**A**), PL (**B**) and Cg (**C**) of 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ pups showing a significant increase of 5-HT fibers in all mPFC subdomains of 5-HTT$^{-/-}$ animals. (**D**) Enlargements of cryosections of P6 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rat brain showing dorsal VTA immunostained for TH (white) with more aberrant fibers leaving the VTA in 5-HTT$^{-/-}$ animals. Graphs in **A**, **B** and **C** show average total length of 5-HT-positive fibers per bin ± SEM. One-way ANOVA ($\alpha = 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



Supplemental Fig. 2. Prefrontal cytoarchitecture is affected in the absence of the 5-HTT. (**A, B, C**) Quantification of total number of Cux1-positive (**A**), Tb1-positive (**B**) and DAPI-positive **(C)** neurons over the complete length of the prefrontal swatch in IL, PL and CG, showing the total number of Cux1-positive and Tbr1-positive cells decrease in the 5-HTT$^{-/-}$ animals **(A, B**) the total number of DAPI-positive cells remain unchanged when we compare 5-HTT$^{+/+}$ with 5-HTT$^{-/-}$ animals (**D**), confirming our significant results in **A** and **B** are not due to the smaller number of cells in the mutant animal 5-HTT$^{-/-}$. Graphs in **A**, **B** and **C** show average number ± SEM. One-way ANOVA ($\alpha = 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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4

Developmental switch in anxiety-like behavior and coinciding prefrontal aberrations in serotonin transporter knockout rats

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In preparation for submittance

Abstract

Besides the known functions of serotonin (5-HT) in key cognitive abilities and emotional regulation, a more complex role for this neurotransmitter during development has now been acknowledged. Serotonergic neurons send their projections to the medial prefrontal cortex (mPFC) and disturbances in the serotonergic system can cause mPFC developmental changes that have lifelong consequences, including increased anxiety. Therefore, more insight into the relationship between serotonergic system development in the mPFC and the appearance of anxiety would be informative. Here, we investigated anxiety-like behavior and mPFC anatomical correlates at adolescent and early-adult ages in 5-HT transporter knockout (5-HTT$^{-/-}$) rats. We find that the 5-HTT$^{-/-}$-induced increase in anxiety is not observed at postnatal day (PND)17 and PND25 and emerges only at PND35 and then persists into adulthood. At PND35, but not at PND17, we found a number of molecular and cellular alterations in the mPFC of 5-HTT$^{-/-}$ animals, including neuronal loss through activation of Caspase-3 as well as an increased serotonergic innervation in the prelimbic and cingulate cortices. In conclusion, 5-HTT-deficient rats show an altered cytoarchitecture in the developing mPFC and increased anxiety-like behaviour that only emerges during adolescence.

Keywords: Prefrontal Cortex Development; Serotonergic System; 5-HTT$^{-/-}$ Knockout Rats; Anxiety; Disturbed Behaviour

Introduction

Anxiety is a feeling associated with excessive worry about the future and fears that something can get out of control and end up wrong[1]. It is a natural, protective and instinctive biological reaction that is important for human beings, because it allows the individual to prepare and plan actions to deal with situations that are interpreted by the brain as uncomfortable or threatening[2,3]. A combination of genetic, hormonal and environmental factors within a subjective context may increase anxiety[4].

When an anxiety response starts, a surge of hormones and neurotransmitters is triggered, which prepares the body for a reaction[2,5]. One of the major neurotransmitters involved in this response is the monoamine serotonin (5-HT)[9,10,11] that is released by serotonergic neurons located in the raphe nuclei in the brainstem[42,43]. These neurons send projections to all brain areas involved in the regulation of mood and behavior, including the limbic system and the prefrontal cortex (PFC)[21,22]. Interestingly, 5-HT is one of the first neurotransmitters to appear during embryonic development of the central nervous system (CNS)[72]. During cortical development 5-HT acts as a neurotrophic factor controlling developmental events such as proliferation, migration, differentiation, cell death, formation of synapses and a correct organization of the cerebral cortex[93]. Thus, the regulation of 5-HT homeostasis during brain development and later in life is critical for normal CNS development and maturation, and its dysregulation has been associated with anatomical, functional and behavioral abnormalities[71,9], including anxiety [73,74].

One key gene variant mediating individual differences in anxiety is the serotonin transporter (5-HTT)-linked polymorphic region (5-HTTLPR)[9,10,11], which regulates the expression of the 5-HTT gene (SLC6A4)[75,76]. Compared to the long (l) allelic variant, the short (s) variant is associated with reduced transcription of the 5-HTT gene and confers increased anxiety[62]. Genetic vulnerability may manifest itself in subjective markers of pathological anxiety only when triggered by environmental factors such as stress[77,78].

To understand anxiety-like behavior and its molecular and cellular underpinnings, animal studies are essential. These studies allow measurements of behavioral responses caused by exposure to potentially anxiogenic situations under controlled laboratory conditions[79]. For instance, 5-HTT knockout (5-HTT$^{-/-}$) rodents are known to display anxiety-related responses when exploring a novel environment[48]. Furthermore, animals allow

more detailed understanding of genotype effects on brain neurocircuitry that may explain 5-HT-dependent anxiety responses. The anxiety response is strongly dependent on the medial PFC (mPFC)[21,22], most likely through its reciprocal connections with the amygdala and other structures of the limbic system[37,38,39] as well as by regulating the monoaminergic nuclei in the brainstem such as the dorsal raphe nucleus (DRN)[42,43]. In turn, serotonergic projections from the DRN regulate the mPFC, thus creating a feedback loop[49,50,51]. In humans, functional magnetic resonance imaging studies have found increased amygdala and cingulate cortex, limbic regions critical for processing of negative emotion, reactivity to fearful stimuli in healthy subjects carrying the s-allele[80,81,85,83], as well as reduced gray matter volume in [81,86,83]. Furthermore, the genetically induced absence of 5-HTT in rodents causes increased serotonergic and catecholaminergic innervation, and alterations in the cytoarchitecture of the subareas of the mPFC, such as the infralimbic, prelimbic and cingulate cortices[58,59]. However, the mechanisms underlying the effects of reduced or lack of 5-HTT, and consequently increased mPFC 5-HT (and dopamine) levels, on cortical cytoarchitecture and anxiety are still unclear. Since inherited 5-HTT down-regulation in animals, and likely also in humans, is associated with a lifelong increase in CNS 5-HT levels, and the function of 5-HT changes over time from exerting neurotrophic actions to being a neurotransmitter, anxiety may have an origin in 5-HT-mediated neurodevelopmental effects on PFC cytoarchitecture.

Here, we aimed to investigate the effect of the absence of the 5-HTT on anxiety and on mPFC cytoarchitecture during a specific developmental time window. To this end, we used 5-HTT knockout rats and wild-type controls to dissect how anxiety develops across juvenile, adolescent and adult stages. To investigate the underlying structural changes in the mPFC, we assessed across these stages the cytoarchitecture and furthermore focused on the serotonergic innervation of this brain area.

Material and methods

Animals

All animal use and care were in accordance with institutional and European guidelines and approved by the Committee for Animal Experiments of Radboud University (Nijmegen, The Netherlands). Wild-type Slc6a41Hubr (5-HTT$^{+/+}$), heterozygous Slc6a41Hubr (5-HTT$^{+/-}$) and knockout Slc6a41Hubr (5-HTT$^{-/-}$) rats were generated by ENU-induced

mutagenesis with Wistar genetic background, as described by Smits *et al.*[70]. All animals were bred at the Central Animal Laboratory of Radboud Nijmegen University (Nijmegen, The Netherlands). Breeding animals were derived from heterozygous knockout rats (5-HTT$^{+/-}$) after 15 generations. Experimental animals were derived from homozygous reproduction. Behavioural and molecular experiments were performed on male animals on postnatal days (PND) 17, 25, 35 and 70. Animals were group-housed (2-4 animals per cage) in standard Macrolon® type 3 cages in temperature-controlled rooms (21°C ± 1°C) and fed with food and water *ad libitum* and were kept in a light/dark cycle of 12:12 h (lights on at 06:00 h). After the behavioural tests, the animals were decapitated and processed as described below.

Elevated Plus Maze (EPM)

To measure anxiety-like behaviour, the animals were housed (2 animals per cage) and handled in their cages for 5 minutes for at least 5 days prior to the test to avoid stress reactions that could interfere with the results. The behaviour testing room was soundproof and the illumination level was maintained at 100 lux. The EPM is an apparatus with two open arms (50 × 10 cm, light intensity 12.1 lux) and two enclosed arms (50 × 10 × 40 cm, light intensity 4.5 lux) both made of polyvinyl chloride and elevated to a height of 50 cm. Rats of PND17, PND25, PND35 and PND70 were placed in the center of the EPM facing the north open arm and allowed to explore the maze for 5 min. Exploratory behaviour on the EPM was recorded using a video camera attached to a computer, which is controlled by a remote device, and EthoVision XT9 Tracking System Noldus software (Wageningen, The Netherlands). After each trial, all arms and the center area were cleaned with super hypochlorous water. The number of entries (an entry is defined as the center of mass of the rat entering the arm) into each arm and the time spent in the open arms were recorded and these measurements serve as an index of anxiety-like behaviour.

Immunohistochemical analyses

After the behavioural test, the animals were sacrificed by decapitation and the brains were rapidly dissected and fixed by overnight immersion in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4 at 4˚C. After fixation, the brains were cryoprotected in 30% sucrose in PBS until

saturated, and then embedded in an M-1 embedding matrix (Shandon, Thermo Fisher Scientific Inc., Waltham, MA, USA) and frozen on dry ice in a plastic cup and stored at -80°C. The cryostat coronal sections were cut at 16 µm, assembled as a series of 10 on Superfrost Plus slides (Thermo Fisher Scientific), air dried and stored desiccated at -20°C. The cryosections were stained immunohistochemically overnight at 4˚C with primary antibodies using mouse anti-NeuN (1:500, Merck Millipore, Bedford, MA, USA; MAB377), rabbit anti-cleaved-Caspase-3 (cl-Casp3, 1:500, Cell Signaling Technologies, Danvers, MA, USA), rabbit anti-5-HT (1:500, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands, S5545) and mouse anti-Satb2 (1:500, Abcam, Cambridge, United Kingdom, ab150502) all diluted in blocking buffer (BB, 5% NGS/NDS/NHS, 0.1% D-lysine, 1% BSA, 1% glycine and 0.4% Triton X-100). After overnight incubation, the sections were washed in PBS and incubated with corresponding species-specific Alexa-conjugated secondary antibody (1:500, Thermo Fisher Scientific) in BB for 30 minutes at room temperature. After washing in PBS, the sections were co-stained with fluorescent 4′,6-diamidino-2-phenylindole (DAPI, 1:1000, Thermo Fisher Scientific, 62248) diluted in PBS for 15 minutes, washed extensively in PBS and embedded in 90% glycerol in PBS. For visualization, a Leica DMI6000B automated high-content fluorescence microscope with the DFC340FX camera and LASAF software was used.

All analyses were performed blindly in at least 4 sequential coronal sections of each mPFC subarea of at least 3 animals of each genotype, which were photographed for cell and fiber quantification. The area which we analyzed in the subdomains of the mPFC was obtained using a 0.1 mm wide square extending from the white matter to the marginal zone, thus including all cortical layers of 5-HTT$^{+/+}$, 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ rats. This rectangle was subdivided into 10 equal bins, in which bin 1 represents the deepest layer and bin 10 the most superficial layer. For counting the cells, we used Photoshop CS6 (Adobe) tools and marker-positive cells were normalized (percentage of total number of DAPI-positive cells or total area of each bin) obtaining a mean for each animal. To better visualize and compare serotonergic innervation in wild-type and mutant mPFC subareas, individual fiber reconstructions of two to four consecutive sections of each animal were obtained bilaterally using NeuronJ, the ImageJ software plugin (National Institutes of Health, Bethesda, USA).

Statistical analysis

Molecular and behavioral data were statistically analyzed by one-way ANOVA (α = 5%) with Bonferroni multiple comparison testing to analyze significant differences between multiple groups using GraphPad Prism 6 (San Diego, California). Data are expressed as means ± SEM.

Results

Absence of 5-HTT results in anxiety-like behavior in an age-dependent manner

In order to examine anxiety-like behavior longitudinally in the absence of 5-HTT, 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats were subjected to the EPM. In the EPM test, the time spent in the open arm was measured for the rats at the ages of PND17, PND25, PND35 and PND70 (Fig. 1). The mean time spent, in seconds, by animals in the 5-HTT$^{-/-}$ group was 24.75s ± 9.3 *versus* 14.47s ± 5.2 in the 5-HTT$^{+/+}$ group at PND17 (Fig. 1E). In PND25 animals, the mean open arm time in the 5-HTT$^{+/+}$ group was 7.71s ± 2.5 seconds *versus* 12.62s ± 3.9 seconds in the 5-HTT$^{-/-}$ group (Fig. 1F). Thus, no significant differences in the time spent in the open arm were found between the two genotypes at the ages of PND17 ($F_{(3.164,9.9)}$ = 0.1013; p = 0.35) and PND25 ($F_{(2.440,9.9)}$ = 0.2001; p = 0.31; Fig. 1E-F). Yet, 5-HTT$^{-/-}$ rats did show a significantly higher distance moved ($F_{(1.623,9.9)}$ = 0.0011; p = 0.019) in the EPM test when compared to the control group at PND25 (Fig. 1I). Anxiety-like behaviour was therefore significantly different between 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ animals at the ages of PND35 ($F_{(19.25,9.9)}$ = 0.0002; p = 0.0056) and PND70 ($F_{(20.99,9.9)}$ = 0.0001; p = 0.0078)(Fig. 1G-H). No significant differences were found between 5-HTT$^{+/-}$ and 5-HTT$^{+/+}$ animals at the ages of PND25, PND35 and PND70 in EPM test parameters evaluated (Supplemental Fig.1). Together, the data suggest increased anxiety-like behaviour in 5-HTT knockout animals at the ages of PND35 and PND70, but not at PND17 and PND25 when anxiety tended to be lower than in the control situation.

Absence of 5-HTT affects the cytoarchitecture of developing mPFC subareas

To identify any cellular changes in the mPFC underlying the age-dependent differences in anxiety-like behavior in 5-HTT$^{-/-}$ *versus* 5-HTT$^{+/+}$

rats, we first immunostained cryosections from mPFC subareas with DAPI in rats of PND17 and PND35 (Fig. 2), two ages at which the EPM data are most distinct from one another. At the age of PND17, we found no significant differences in the number of DAPI-positive cells across layers of infralimbic ($p = 0.68$, Fig. 2E and Supplemental Fig. 2D), prelimbic ($p = 0.91$, Fig. 2F and Supplemental Fig. 2E) and cingulate ($p = 0.52$; Fig. 2G and Supplemental Fig. 2F) subareas of the mPFC in 5-HTT$^{-/-}$ *versus* wild-type rats. However, at PND35 we observed a significant decrease in DAPI-positive cells in the deep layers of the prelimbic cortex (bin1, $p = 0.0034$; bin2, $p = 0.0031$; bin6, $p = 0.023$; Fig. 2I) and cingulate (bin3, $p = 0.012$; bin5, $p = 0.024$; bin6, $p = 0.026$; Fig. 2J) of the 5-HTT$^{-/-}$ rats compared to controls. When we evaluated the cortical thickness of the mPFC subdomains, we found a significant increase in the cingulate cortex of the PND35 5-HTT$^{-/-}$ group when compared to 5-HTT$^{+/+}$ rats ($p = 0.0036$; Fig. 2D), but not between the genotypes in infralimbic (Fig. 2B) and prelimbic (Fig. 2C) areas at the ages analysed. Neuronal number assessments using NeuN as a marker revealed only at PND35 a significant decrease in the number of mature neurons in the deep cortical layers of the infralimbic (bin5, $p = 0.033$; Fig. 3F) and cingulate (bin3, $p = 0.041$; Fig. 3H) cortices and deep and superficial layers of the prelimbic cortex (bin1, $p = 0.0019$; bin2, $p = 0.0019$; bin5, $p = 0.034$; bin10, $p = 0.0020$; Fig. 3G) of the 5-HTT$^{-/-}$ animals when compared to controls. No significant differences were found in the number of mature neurons per area among the groups analyzed at PND17 (Fig. 3C-E, I-K). To investigate the levels of programmed cell death, a significant increase of the cleaved-Caspase-3 (cl-Casp3) cell death marker was observed in all cortical layers of the infralimbic ($p = 0.00039$; Fig. 4C and I), prelimbic ($p = 0.000028$, Fig. 4D and J) and cingulate ($p = 0.000011$; Fig. 4E and K) cortices in animals of the PND17 5-HTT$^{-/-}$ group. At PND35, a significant increase in cl-Casp3-positive cells was found in the superficial layers of the infralimbic cortex (bin9, $p = 0.024$, Fig. 4F and I), prelimbic (bin6, $p = 0.0095$, bin9, $p = 0.047$, Fig. 4G and J) and in all layers of the cingulate cortex ($p = 0.00038$; Fig. 4H and K) of 5-HTT$^{-/-}$ animals. Furthermore, PND35 5-HTT$^{-/-}$ rats showed a significant increase in the number of apoptotic neurons in all subareas of the mPFC (Supplementary Fig. 2A-C) at PND35. Interestingly, not all cl-Casp3-positive cells colocalized with NeuN-positive cells (Fig. 4A-B), suggesting apoptosis of an additional cell type other than neuronal. Together, the data suggest that loss of the 5-HTT results in cellular differences across layers in all subareas of the mPFC and that these disturbances are more pronounced in PND35 than PND17 animals.

The fact that we found changes in the total number of mature neurons in all mPFC subareas tested brought us to the question of how these changes could impact the structure of the cortical layers and which specific layer would be more affected. To address this question, we used the layers II-V marker Satb2. Immunostaining of PND17 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ cryosections showed no significant differences between the two groups in infralimbic ($p = 0.34$), prelimbic ($p = 0.85$) and cingulate ($p = 0.14$) cortices (Fig. 5A-K), while in PND35 5-HTT$^{-/-}$ animals a significant increase in the superficial layer of the prelimbic cortex (bin 8, $p = 0.011$; Fig. 5G) was observed.

Absence of 5-HTT causes disturbances in serotonergic innervation of mPFC subareas

Previous studies have demonstrated an important role of the serotonergic system in the development of the mPFC[58,59]. Furthermore, proper PFC functioning is required for the afferent control of the DRN, thus regulating the 5-HT supply during a stress response[42,43]. When we measured the number of positive serotonergic fibers in mPFC cryosections, we found a significant increase in the lengths of these fibers in the infralimbic ($p = 0.025$, Fig. 6C and I), prelimbic ($p = 0.000065$, Fig. 6D and J) and cingulate ($p = 0.000024$, Fig. 6E and K) cortex of PND17 5-HTT$^{-/-}$ animals. However, at PND35 this significant increase was identified only in the prelimbic ($p = 0.000012$; Fig. 6G and J) and cingulate ($p = 0.015$, Fig. 6H and K) cortices of the 5-HTT$^{-/-}$ animals. Together, the data suggest that in the absence of the 5-HTT, serotonergic innervation of the mPFC subareas is increased at PND17 and PND35.

Discussion

In this study, we demonstrate that in 5-HTT$^{-/-}$ rats increased anxiety emerges in a defined age window, namely between PND25 and PND35. We also demonstrate that changes in cortical cytoarchitecture and cell death in mPFC subdomains of adolescent and adult 5-HTT$^{-/-}$ rats. The structural changes were observed in the infralimbic, prelimbic and cingulate cortices; a decrease in the total number of cells was found, mainly in layers II-V. Furthermore, the absence of the transporter interfered with the degree of neuron maturation and led to an increase of cleaved-Caspase-3-positive cells suggesting increased programmed cell death. Finally, we found an increase

in serotonergic innervation of all subareas of the mPFC in 5-HTT$^{-/-}$ animals, indicating a strong genotype effect on cortical development and organization via modulation of 5-HT levels.

We used the EPM test to measure anxiety in 5-HTT$^{-/-}$ and wild-type controls across ages. We observed increased anxiety in adolescent and adult, but not juvenile 5-HTT$^{-/-}$ rats. Heterozygous 5-HTT$^{+/-}$ rats behaved like 5-HTT$^{+/+}$ rats. The absence of anxiety in juvenile 5-HTT$^{-/-}$ rats may be due to a delay in brain development caused by increased brain 5-HT levels. Excess of cortical 5-HT affects the migration speed and positioning of pyramidal glutamatergic neurons[116] and GABAergic interneurons[94] in the superficial embryonic cortical layers of 5-HTT$^{-/-}$ knockout mice. Owing to its high functional complexity and the large number of connections with various subcortical areas[100], PFC maturation is relatively late. Therefore, a delay in cortical development due to a slower speed of excitatory and inhibitory neuron migration may particularly affect the PFC cytoarchitecture and PFC-dependent behaviours.

Morphogenic responses to the change in 5-HT levels may vary enormously, depending on the genetic polymorphism as well as the 5-HT receptor subtypes expressed by different target cells[104,105]. Serotonergic receptors located on neural progenitor cells induce proliferation, and maturation of postmitotic neurons, cell survival as well as the release of astroglial neurotrophic factors that also influence neuronal maturation[106,112,113]. For example, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors are expressed on astrocytes and neurons and regulate, among other functions, c-AMP levels and calcium availability, which alters the internal cytoskeleton and results in cell proliferation, synaptogenesis and apoptosis[104]. In 5-HTT$^{-/-}$ animals, the expression of these receptors is deregulated[105]. The alteration in 5-HT levels during embryonic rat development affects the maturation of pyramidal neurons and causes a reduction of dendritic arborization in the somatosensory cortex[95], and alters neurite outgrowth in the neocortex of 5-HTT$^{-/-}$ mice[117]. Furthermore, apoptotic pathways are activated, as demonstrated by an increased number of cleaved-Caspase-3-positive cells in all mPFC subareas, presumably explaining the significant reduction in mature neurons. Interestingly, the death of neurons was more pronounced at PND35 than at PND17, which may contribute to the emergence of increased anxiety at PND35 in the 5-HTT$^{-/-}$ rats.

Reduced levels or the absence of the 5-HTT in humans and rodents alter cortical characteristics in the adult[115]. Although, in contrast to the S1[79,90-92] of 5-HTT$^{-/-}$ rats, the cortical thickness of the mPFC subdomains appears

normal, various cortical layers showed significant changes in expression of certain markers such as Satb2 and Cux1[119,58,59]. Some of these alterations may be due to changes in the positioning of interneurons and pyramidal neurons within the superficial cortical layers[94,116].

The structural abnormalities and functional consequences found in the absence of the 5-HTT probably result from the elevated extracellular 5-HT levels during critical developmental time periods[109]. Extracellular 5-HT concentrations are markedly increased in the striatum and cortex of 5-HTT$^{+/-}$ and 5-HTT$^{-/-}$ mice[96,114]. Measurements of serotonergic axon length and number of axonal varicosities by quantitative immunocytochemistry have shown an increase in the density of serotonergic innervation in various brain regions of 5-HTT$^{-/-}$ animals[58,59,115], in line with our results in the mPFC. Serotonergic hyperinnervation of not only subdomains of the mPFC, but also the amygdala-hippocampal nucleus and ventral hippocampus may be responsible for the observed anxious behavior[115,120]. Neuroimaging studies in children and adolescents exposed to stressful situations have demonstrated alterations in amygdala-mPFC connectivity, increased depression, attention problems, and aggressive and anxiety-like behavior[121], and these poor connections persist until adulthood[125,126]. Early-life stress events in rodents also alter mPFC-amygdala activity in adult life[123,124].

A limitation of this work is that the molecular changes associated with behavior have been identified only in a specific developmental time window and not at all ages. Yet, our previous studies on 5-HTT$^{-/-}$ rats at early-postnatal ages have demonstrated delayed cortical development, and altered identity of deep and superficial cortical layers in the mPFC[58,59]. We now find that these alterations last throughout adulthood, despite the appearance of possible compensation mechanisms with an increase in Satb2 expression between the ages of PND6[58,59], PND17 and PND35.

In conclusion, absence of the 5-HTT gene in rats causes anxiety-like behavior between PND25 and PND35. The associated neuroanatomical changes in the mPFC involve serotonergic hyperinnervation and a significant loss of neurons. The latter is possibly due to activation of cellular apoptosis pathways in the prelimbic and cingulate cortices of 5-HTT$^{-/-}$ animals. Since the death of neurons coincides with increased serotonergic innervation in the cingulate cortex and the emergence of an anxiety-like phenotype in 5-HTT$^{-/-}$ rats, a neuronal excess earlier in life may protect against this phenotype.

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Conflict of interest

The authors declare that they have no conflict of interest.

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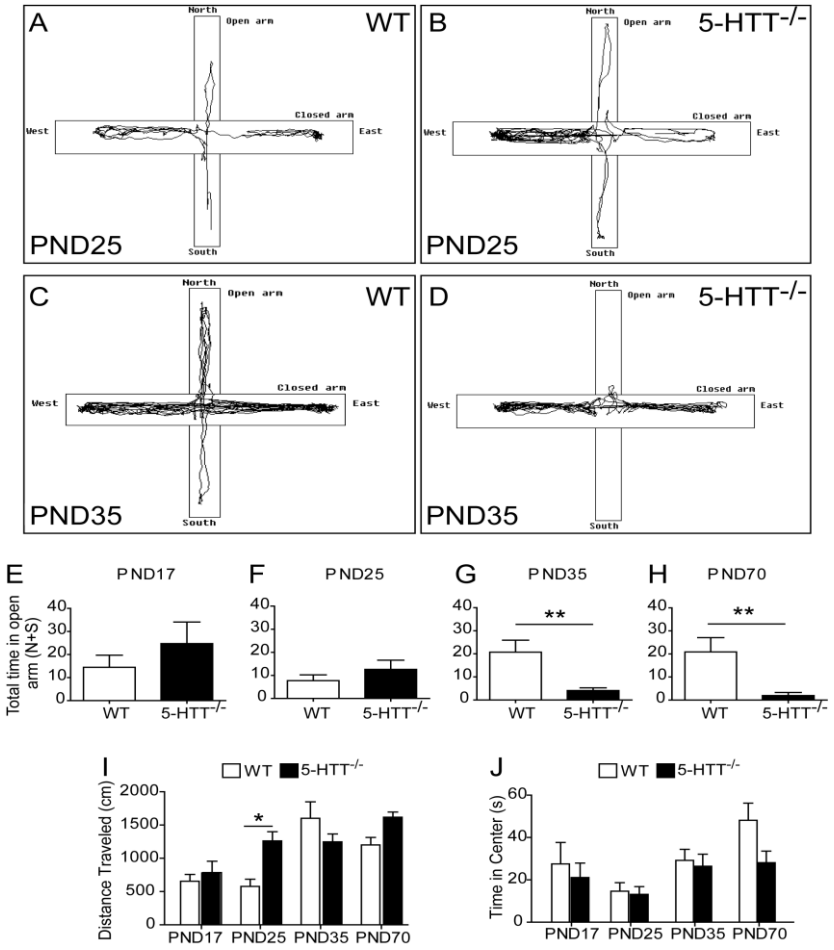


Fig. 1. The absence of the serotonin transporter (5-HTT) affects anxiety-like behavior in the elevated plus-maze (EPM) test in an age-dependent manner. **(A-D)** Movement traces by EthoVision XT9 Tracking) of 5-HTT$^{-/-}$ and wild-type (WT) rats at PND25 and PND35. **(E-H)** Anxiety-like behavior identified by the total time spent in the open arms at PND35 **(G)** and PND70 **(H)** and not at PND17 (**E**) and PND25 (**F**). **(I)** Total distance traveled data showed an increase of mobility in 5-HTT$^{-/-}$ rats compared to controls at PND25. **(J)** No differences were found between the genotypes in the total time spent in the center. Data show mean (± SEM), *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA. (5-HTT$^{-/-}$, n = 10; WT, n = 10).

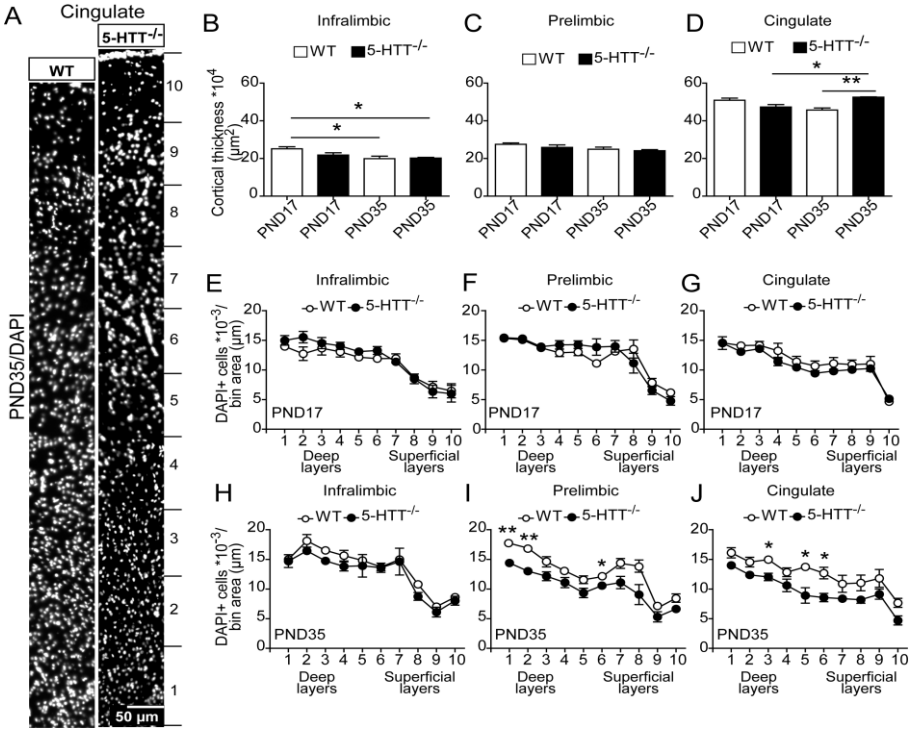


Fig. 2. The absence of 5-HTT changes the cortical thickness of the mPFC resulting in a significant decrease in the total number of DAPI+ cells in the deep cortical layers in an age- and subdomain-dependent manner. **(A)** Staining of cryosections of cingulate cortex at PND35 in 5-HTT$^{-/-}$ and wild-type (WT) rats, showing DAPI-positive cells (white). **(B-D)** Measurement of cortical thickness showing a significant increase in cingulate cortex thickness in 5-HTT$^{-/-}$ animals at PND35 when compared to WT rats. (E-G**)** No significant differences in the total number of cells at PND17 between 5-HTT$^{-/-}$ and WT in all mPFC subareas. **(H-J)** A significant decrease in the total number of cells in deep layers of prelimbic and cingulate cortices, but not infralimbic, in 5-HTT$^{-/-}$ animals at PND35, when compared to the WT group. Data show mean (± SEM) number of cells, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA (5-HTT$^{-/-}$, $n = 3$; WT, $n = 3$).

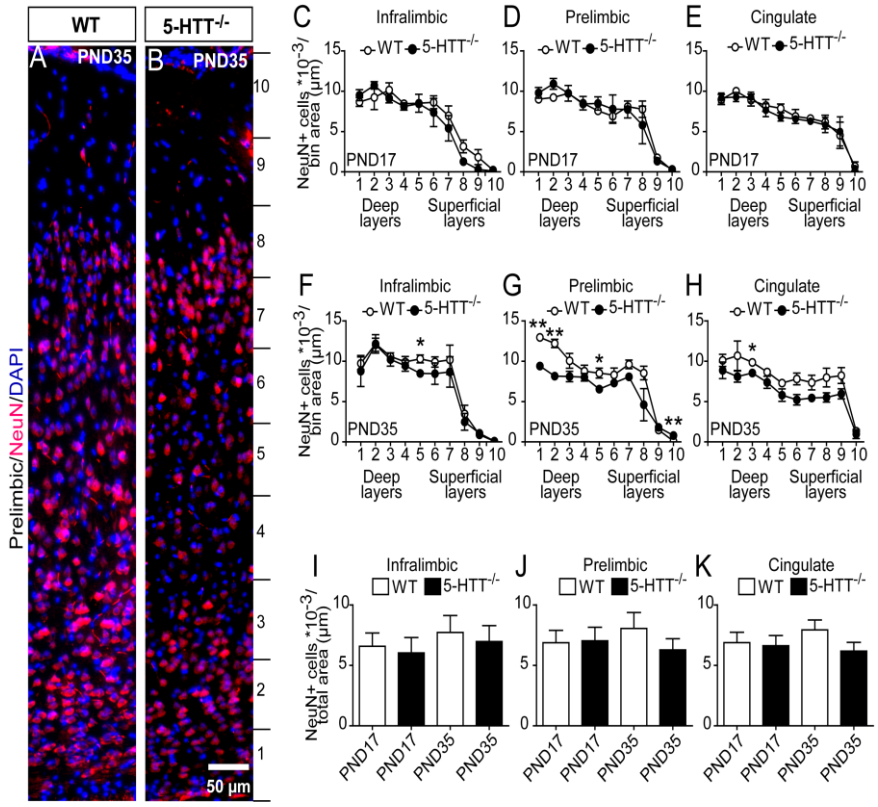


Fig. 3. The absence of 5-HTT decreases the number of mature neurons in all subareas of the mPFC. **(A, B)** Immunostaining showing NeuN-positive cells (red) in prelimbic cryosections of 5-HTT$^{-/-}$ and WT brains at PND35 counterstained with DAPI (blue). **(C-E)** Quantification of mature neurons (NeuN^{+}) showing no significant differences between 5-HTT$^{-/-}$ and wild-type (WT) rats at PND17. **(F-H)** A significant decrease in NeuN-positive neurons in deep and superficial layers of 5-HTT$^{-/-}$ rats when compared to WT at PND35. **(I-K)** No significant differences were found in the total number of NeuN-positive neurons in total mPFC subarea of 5-HTT$^{-/-}$ animals when compared to WT. Data show mean (± SEM) number of neurons, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA (5-HTT$^{-/-}$, n = 3; WT, n = 3).

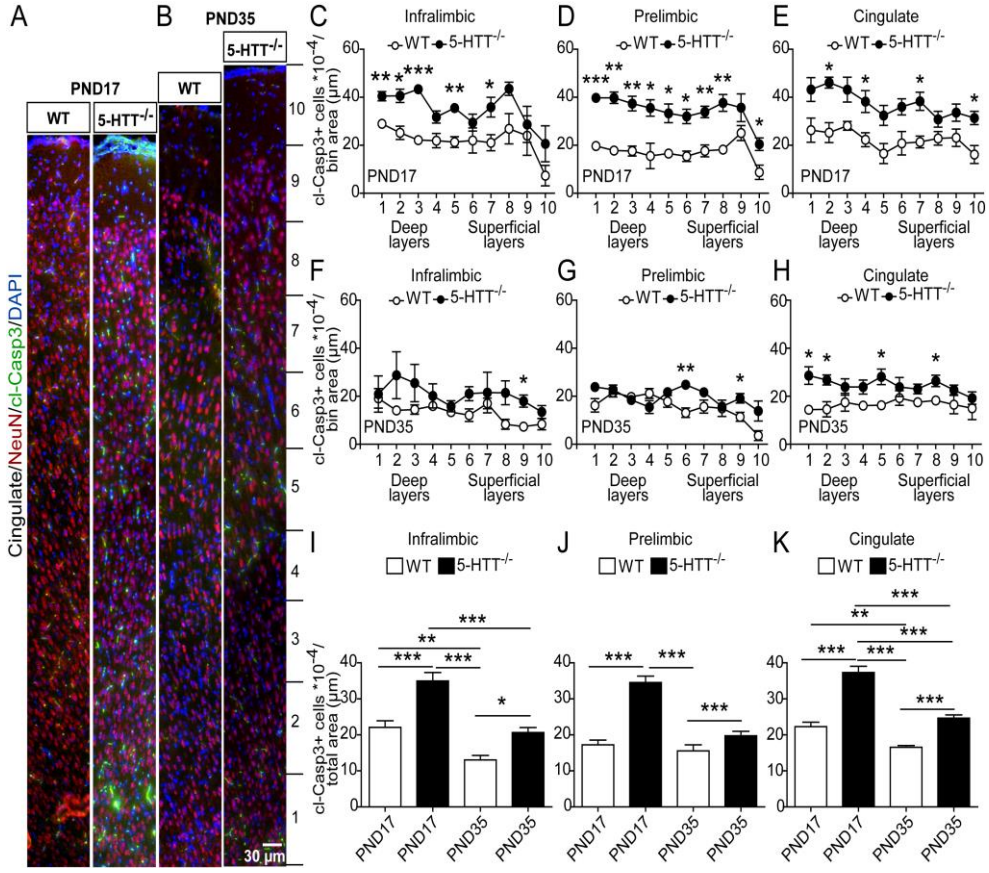


Fig. 4. The absence of 5-HTT increases number of apoptosic cells in mPFC subareas. **(A, B)** Immunostaining showing cl-Casp-3- and NeuN-positive cells and their co-expression in the cingulate cortex at PND17. (**C-E)** Quantitative results showing a significant increase in cl-Casp3-positive cells in deep and superficial layers of the infralimbic, prelimbic and cingulate cortices of 5-HTT$^{-/-}$ animals when compared to wild-type (WT) at PND17 and PND35 **(F-H). (I, K)** Significant increase of cl-Casp3-positive cells in all mPFC subareas of 5-HTT$^{-/-}$ rats when compared with WT at PND17 and PND35. Data show mean (± SEM) number of cells, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (5-HTT$^{-/-}$, n = 3; WT, n = 3).

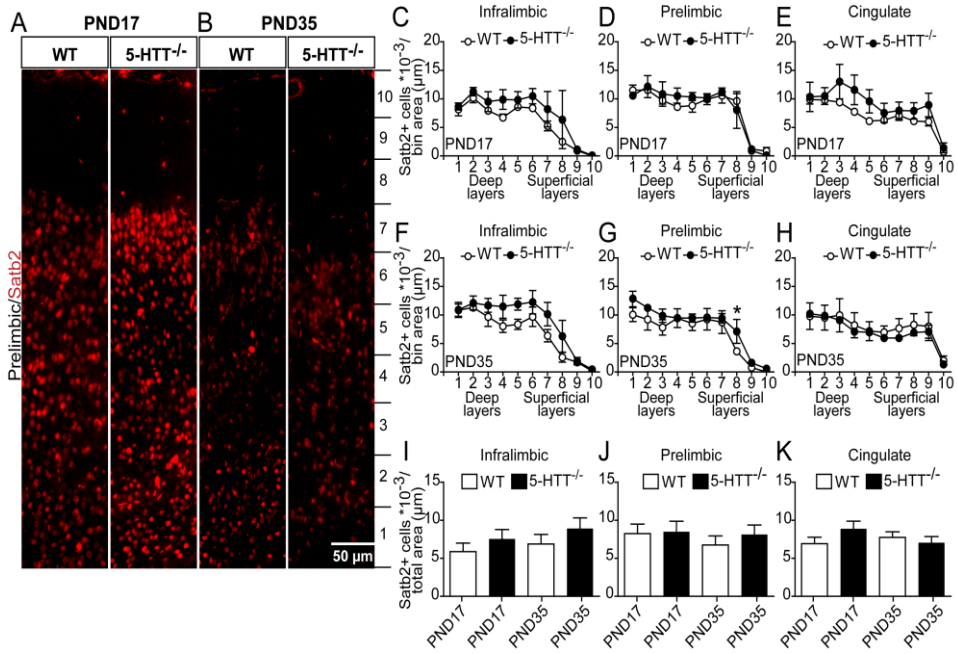


Fig. 5. The absence of 5-HTT affects prefrontal cytoarchitecture. **(A, B)** Qualitative data showing immunostaining for Satb2 in cryosections of the prelimbic cortex at PND17 and PND35 in 5-HTT$^{-/-}$ and wild-type (WT rats. **(C-H)** No significant differences were found in the total number of Satb2-positive cells of 5-HTT$^{-/-}$ compared to WT animals except for bin8 (superficial) of the prelimbic at PND35. **(I, K)** No significant differences were found in the total number of Satb2-positive cells per subareas at PND17 and PND35. Data show mean (± SEM) number of cells, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (5-HTT$^{-/-}$, n = 3; WT, n = 3).

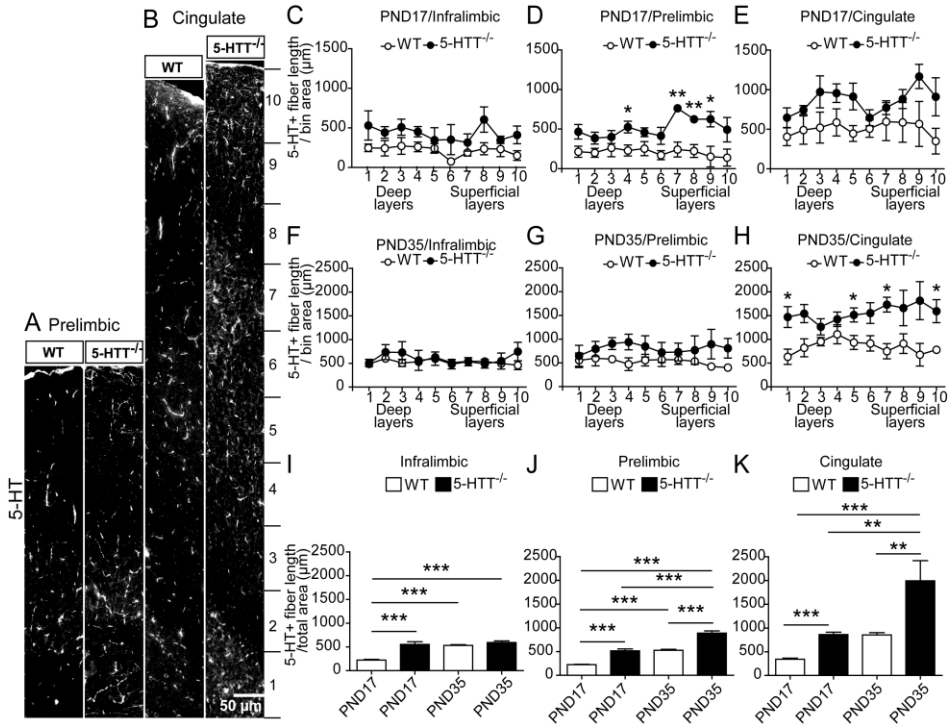
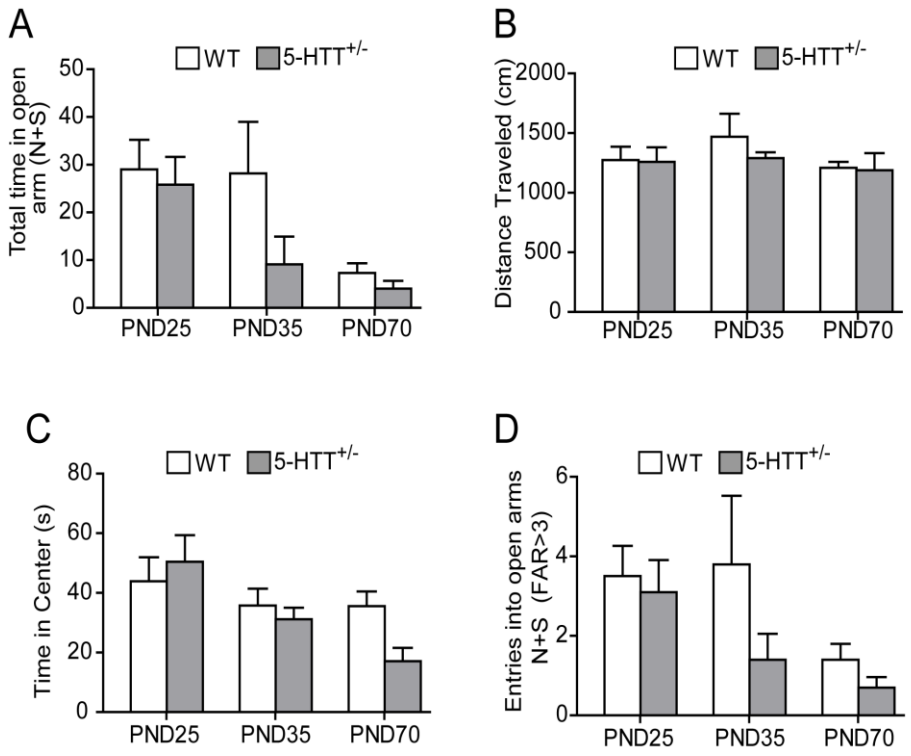
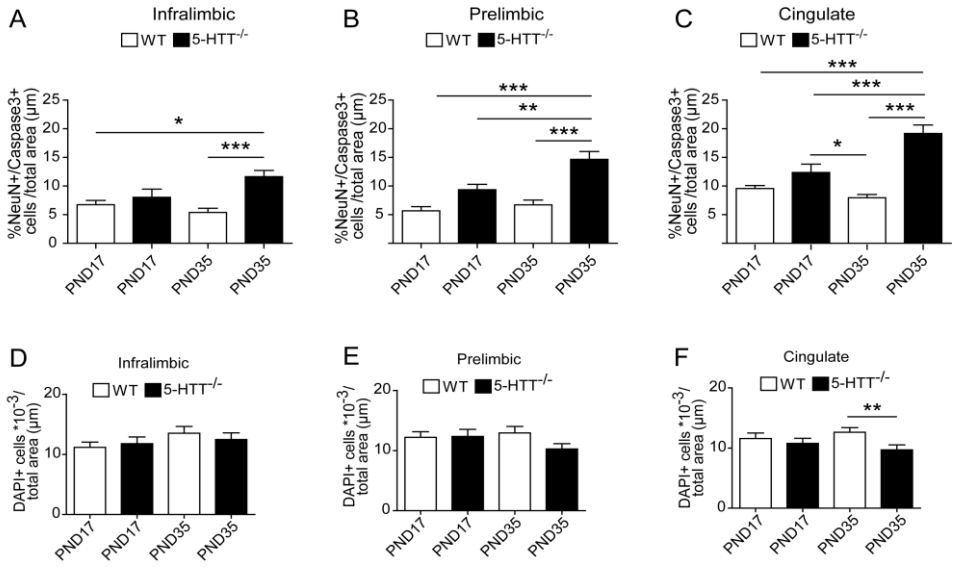


Fig. 6. The absence of 5-HTT affects mPFC serotonergic innervation. (**A, B****)*** Immunostaining for 5-hydroxytryptamine (5-HT, white) of cryosections of the prelimbic cortex of 5-HTT$^{-/-}$ and wild-type (WT) animals at PND17 and cingulate cortex at PND35. **(C-E).** A significant increase of serotonergic innervation in deep and superficial layers in the prelimbic cortex of 5-HTT$^{-/-}$ rats when compared to WT at PND17. **(F-H)** A significant increase in serotonergic innervation in deep and superficial layers of the cingulate cortex of 5-HTT$^{-/-}$ rats when compared to WT at PND35, but not in infra- and prelimbic cortices. **(I-K)** A significant increase of fiber length per total area of the infralimbic, prelimbic and cingulate cortices at PND17 in absence of 5-HTT. However, this was significant only in the prelimbic and cingulate cortices at PND35. Data show mean (± SEM) fiber length, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA (5-HTT$^{-/-}$, n = 3; WT, n = 3).



Supplemental Fig. 1. Elevated plus-maze (EPM) test parameters on anxiety-like behavior in 5-HTT$^{+/-}$ and WT rats. No significant differences were found between 5-HTT$^{+/-}$ and wild-type (WT) rats in the total time spent in the open arm **(A)**, total distance traveled **(B)**, total time spent at center **(C)**, and number of entries into open arms **(D)** at PND25, PND35, and PND70. Data show mean (± SEM), *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (5-HTT$^{+/-}$, n = 10; WT, n = 10).



Supplemental Fig. 2. The absence of 5-HTT increases apoptosis in neurons of the mPFC. **(A-C)** Quantification of the percentage of $Casp3^{+}/NeuN^{+}$ cells showing a significant increase in mature neuron death in infralimbic, prelimbic and cingulate at PND35. **(D-F)** The total number of cells per total mPFC subarea, showing a significant decrease in the number of DAPI positive cells in 5-$HT^{-/-}$ animals at PND35 when compared to wild-type (WT) rats. Data show mean (± SEM), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA (5-$HTT^{-/-}$, n = 3; WT, n = 3).

5

Neurodevelopmental and behavioral consequences of perinatal exposure to the HIV drug efavirenz in a rodent model

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Abstract

Efavirenz is recommended as a preferred first-line drug for women of childbearing potential living with human immunodeficiency virus. Efavirenz is known for its central nervous system side effects, which are partly mediated by serotonergic actions. The neurotransmitter serotonin exerts neurotrophic effects during neurodevelopment and antenatal exposure to serotonergic agents has been linked to developmental delay. Although the teratogenic risks of efavirenz appear to be minimal, data on long-term developmental effects remain scarce. Here, we aimed to investigate the short- and long-term behavioral and neurodevelopmental effects of perinatal efavirenz exposure. We treated pregnant rats from gestation day 1 until postnatal day 7 with efavirenz (100 mg/kg) or vehicle. We measured behavioral outcomes in male offspring during the first three postnatal weeks, adolescence and adulthood, and conducted brain immunohistochemistry analyses after sacrifice. Perinatal efavirenz exposure resulted in reduced body weight and delayed reflex and motor development. During adulthood we observed a decrease in the total number of cells and mature neurons in the motor cortex, as well as an increase in the number of cleaved-Caspase-3-positive cells and serotonergic fibers. Together, our data show a developmental delay and persistent changes in the brain motor cortex of rats exposed to efavirenz perinatally. Because over one million children born annually are exposed to antiretroviral therapy, our findings underline the need for clinical studies on long-term neurodevelopmental outcomes of perinatal exposure to efavirenz.

Keywords: Neurodevelopment, Perinatal, Corticogenesis, Side Effect, Antiretroviral Therapy, Efavirenz, Serotonin

Introduction

Every year, an estimated 1.4 million women living with human immunodeficiency virus (HIV) become pregnant. The use of antiretroviral therapy (ART) during pregnancy, delivery and breastfeeding, successfully reduces the risk of mother-to-child transmission of HIV to less than 5%[1]. The latest interim guidelines of the World Health Organization (WHO) recommend dolutegravir as the general drug of choice for people living with HIV[2]. However, due to concerns about neural tube defects among first-trimester dolutegravir exposures, efavirenz (EFV) remains the preferred option in women of childbearing potential[3]. Research on the safety of EFV during pregnancy has focused largely on infant health shortly after birth[4]. Although risks for gross teratogenicity seem to be minimal, research on long-term neurodevelopmental effects of perinatal exposure to EFV remains scarce[5, 6].

EFV passes through the placenta and is present in breast milk, resulting in detectable concentrations in the blood of fetuses and breast-fed infants[7, 8]. After entry into the blood stream, both EFV and its primary metabolite 8-hydroxy-efavirenz (8-OH-EFV) readily penetrate the cerebrospinal fluid and target various cellular pathways within the central nervous system (CNS), predominantly the serotonergic system[9-12]. For example, EFV acts as a serotonin$(5\text{-HT})_6$ receptor inverse agonist, 5-HT_{2A}, 5-HT_{2C} and 5-HT_{3A} receptor antagonist, and a blocker of the 5-HT transporter (5-HTT)[13]. In rats, EFV preferentially binds to the 5-HT_{2A} receptor[14].

Importantly, 5-HT exerts neurotrophic functions during early development[15, 16]. Increases in brain 5-HT levels, induced by genetic 5-HTT inactivation, have been shown to alter the serotonergic innervation of the prefrontal cortex[17], migration of inhibitory neurons to the neocortex[18], and maturation of the sensory cortex[19]. The latter has also been observed after pharmacological 5-HTT inhibition by prenatal selective serotonin reuptake inhibitor (SSRI) exposure[18, 20]. Both genetic and pharmacological 5-HTT modulation during early development have been associated with a delay in reflex and motor development, disturbed sensorimotor gating, decreased social behavior, and anxiety and depression-like phenotypes[21-25]. Moreover, children perinatally exposed to SSRIs show reduced language and motor development, and a two-fold increased risk of autism spectrum phenotypes[26-28]. Given that EFV particularly targets the serotonergic system, we hypothesized that perinatal EFV exposure might also lead to long-lasting neurodevelopmental consequences.

Here, we aimed to investigate the short- and long-term behavioral and neurodevelopmental effects of perinatal EFV exposure in a rodent model. We conducted a behavioral test battery including tests for reflex development, motor performance, sensorimotor gating and anxiety- and depressive-like behavior, during early life, adolescence, and adulthood. Throughout the treatment period, we monitored maternal care. Because we observed changes in motor behavior, we investigated the cytoarchitecture of the motor cortex to study the underlying cellular mechanisms. Our results indicate that perinatal EFV exposure is associated with neurodevelopmental delay, accompanied by long-lasting changes in motor cortex morphology.

Materials and methods

Animals

Rats used in this experiment were bred in-house from Wistar male breeders and nulliparous Wistar females weighing 185-215 g, purchased from Charles River, Cologne, Germany. After a two-week acclimatization period, female rats were subjected to a timed mating procedure (using Impedance Checker MK-10B, Muromachi Kikai, Tokyo, Japan) as described previously[25]. Pregnancy was determined by observation of a vaginal plug the day after breeding gestational day (GD) 1. Pregnant rats were alternally assigned to daily treatment with EFV or vehicle from GD1 to postnatal day (PND) 7 by order of birth. PND7 resembles the human functional brain maturity around birth[29]. Litters were culled to 10 pups and pups were weaned on PND21. Male offspring from four EFV-exposed (n = 24) and four vehicle-exposed nests (n = 20) were used for experiments. Developmental milestones and behavior were assessed during the first three weeks of life (PND2-21), adolescence (PND34-35), and adulthood (PND69-70). After completion of the last experiments, rats (PND73-75) were sacrificed and their brains were removed for immunohistochemistry analyses. Fig. 1 shows a schematic representation of the experimental timeline. Sample sizes were based on power calculations from previous studies using comparable animal models and similar behavioral analyses [24, 25]. Animals were tested randomly, and both investigators and caregivers were blinded to the groups during experiments and outcome assessment. Blinding was ensured using coded treatment containers of identical appearance. Videos of the reflex and motor performance tests were re-analyzed by a second blinded researcher resulting in similar results. Animals were housed in pairs in standard Macrolon® type

3 cages in temperature-controlled rooms (21°C ± 1°C) under a standard 12-h light/dark cycle (lights on at 07:00 h) with food and water available *ad libitum* (Sniff, long cut pellet, Bio Services, Uden, The Netherlands). Experiments were carried out in accordance with the European Communities Council Directive (2010/63/EU) and approved by the Committee for Animal Experiments of the Radboud university medical center Nijmegen, The Netherlands (ref no. 2012-236).

Drug treatment

EFV or vehicle was administered blindly by oral gavage in a volume of 5 mL/kg. We used a dose of 100 mg/kg, based on previous work demonstrating plasma levels within the human therapeutic range (1.0-4.0 mg/L)[30] and unpublished pilot work. Drug solution was prepared by diluting EFV oral suspension (Stocrin suspension 30 mg/mL, Merck Sharp & Dohme, Haarlem, The Netherlands) with distilled water. As vehicle, we used a 1% cellulose suspension (Genfarma B.V., Maarssen, The Netherlands), enriched with the EFV solution additives, consisting of medium chain triglyceride oil (Newpharma, Liège, Belgium) and strawberry and peppermint essence (Lecocq N.V./S.A., Zonhoven, Belgium).

Measurement of EFV in blood plasma

Ninety minutes (min) after drug administration at GD4, we collected blood, obtained by tail cut, of all pregnant rats (n = 8). Blood was collected in Microvette CB 300 tubes (containing EDTA; Sarstedt, Germany) and centrifuged for 15 min at 4°C with a speed of 4000 rpm. The supernatant (plasma) was stored at -20°C until analysis. Supernatants from the experimental group were used for determination of plasma peak EFV levels using a validated reversed phase ultra-performance liquid chromatography with ultraviolet detection bioanalytical assay, validated for human plasma. Fur the current analysis, quality control samples prepared with blank rat plasma were included to assure validity of the assay. During the bioanalysis, the quality control samples in rat plasma did not deviate more than 15% from the theoretical value.

Maternal care

Maternal care was scored from PND2 to PND9 using a procedure adapted from Ivy et al[31]. Litters were observed during three sessions per day of 75 min each: at 7:30 am, 1:30 pm (light phase) and 7:30 pm (dark phase). Throughout each session, behavior was scored every third min (25 observations per session). The behaviors scored include: (1) nursing more than half of the nest, (2) licking or grooming any pup, (3) spending time out of the nest, (4) self-licking or grooming, and (5) eating or drinking.

General development

Body weight was measured daily during early life (PND2-21), adolescence (PND34) and adulthood (PND70). In addition, we recorded eye-opening every morning from PND13 until both eyes were open. Scores were defined as: (0) both eyes closed, (1) one eye open, (2) both eyes open. An eye was considered "open" if the palpebral fissure was ≥2 mm[32].

Behavioral development

The experimental test battery was adapted from Kroeze et al. and included the following functional domains[25]: reflex development (righting reflex, negative geotaxis and acoustic startle reflex), motor development (swimming performance and bar holding), sensorimotor gating (prepulse inhibition [PPI]), and anxiety- and depressive-like behavior (elevated plus maze and forced swim test).

Reflex development – righting reflex, negative geotaxis, acoustic startle reflex

During the righting reflex test, rats (PND2-10) were placed on their back, while recording the time until they returned to prostate position, with a maximum of 60 seconds (sec)[33]. Negative geotaxis was examined using a 40° inclined wooden plank with a wire mesh. Rats (PND4-14) were placed facing down the slope and allowed to turn 180° within 90 sec[21]. All test sessions were recorded for later rescoring by a second researcher. See below for methods of the acoustic startle reflex.

Motor development – swimming performance, bar holding

During the swimming performance test, rats (PND8, 10, 12, 14, 22) were dropped from ± 20 cm into a basin filled with 27°C water and observed for 5-10 sec. Scoring was based on the position of the nose in the water: 1) entire head underwater, (2) nose underwater, ears partially underwater with the back of head above water level, (3) nose above water level but ears partially underwater, (4) entire head above water level[34]. Bar holding was tested by positioning rats (PND10-21) with their forepaws on a wooden bar (3 mm x 40 cm), suspended 45 cm above the bench surface. The latency to fall from the bar was recorded, with a maximum of 50 sec. If the rat fell immediately, the procedure was repeated up to three times[25, 35].

Acoustic startle reflex and sensorimotor gating - prepulse inhibition

At PND 21, 35 and 70, acoustic startle and prepulse inhibition (PPI) experiments were performed in four acoustic startle chambers (San Diego Instruments, San Diego, USA), according to the methods of Sontag et al.[36]. Acoustic startle response was defined as the mean startle amplitude from the ten test blocks of the startle trials. The percentage of prepulse inhibition was calculated using the formula: 100 – (average of startle amplitude on prepulse trial/average of startle amplitude on startle trial) x 100%[36].

Anxiety-like behavior - Elevated Plus Maze

The elevated plus maze is a polyvinylchloride apparatus with two open (50 x 10 cm, light intensity 12.1 lux) and two enclosed (50 x 10 x 40 cm, light intensity 4.5 lux) arms, elevated at a height of 50 cm[37]. Rats (PND69) were placed in the center of the elevated plus maze facing one of the open arms and allowed to freely explore the maze for 5 min. Movements were measured using the EthoVision XT9 Tracking System, Noldus, Wageningen, The Netherlands.

Depressive-like behavior - Forced Swim Test

During the induction phase, rats (PND69) were placed in cylindrical glass tanks (24 cm diameter x 65 cm height filled to 35 cm with 22°C water) for 15 min. After 24 hours, rats (PND70) were placed in the same tank for 5 min (test phase). Immobility was defined as no movements or minimal

movements necessary to keep the nose above water level for ≥ 2 sec. While slight movements of forepaws or paw placement on cylinder walls were still considered immobility, active climbing, diving, and swimming were scored as mobility[24]. The duration of immobility (sec) was recorded with Observer XT 12.5 (Noldus Information Technology, Wageningen, The Netherlands). Both phases of the forced swim test (induction and test phase) were performed at the end of the testing days (after completion of the other behavioral tests).

Immunohistochemistry

After a minimum of two days after completion of the last behavior experiments, rats (PND73-75) were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (200mg/kg) and perfused transcardially with cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Brains were quickly removed and immersed in 4% PFA for 48 hours. Next, brains were washed in PBS, placed in a 30% sucrose solution, frozen on dry-ice, and stored at -80˚C. A minimum of five brains per treatment group were selected for immunohistochemistry analyses. 16 µm sections were cut on a Microm Cryostat, mounted on Superfrost® Plus slides (Thermo Fisher Scientific, Waltham, MA, USA), air-dried and stored desiccated at -20°C. Cryosections were obtained and stained immunohistochemically as previously described[17], using the following antibodies: mouse anti-NeuN (1:500, Merck Millipore, Bedford, MA, USA; MAB377), rabbit anti-cleaved-Caspase-3 (cl-Casp3, 1:500, Cell Signaling Technologies, Danvers, MA, USA; ASP175), rabbit anti-5-HT (1:500, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands; S5545), and rabbit anti-TH (1:500, Merck Millipore, Bedford, MA, USA; AB152), species-specific Alexa-conjugated secondary antibody (1:500, Thermo Fisher Scientific; A32732, A32723). After washing in PBS, sections were counterstained with fluorescent DAPI (1:1000, Thermo Fisher Scientific; 62248) diluted in PBS for 15 minutes, washed extensively in PBS and embedded in 90% glycerol in PBS. For visualization, a Leica DMRA fluorescence microscope equipped with a DFC340FX camera and LASAF software was used.

Quantification

For quantification of cells and fibers in coronal sections, pictures of at least eight brains per group were acquired similar to Witteveen et al.[38].

Stained cells were counted in radial units of 0.1-mm wide in the presumptive primary motor cortex (M1) of anatomically matched brain sections using Photoshop CS6 (Adobe). The overall cortical length, above white matter, of M1 was divided into 10 equal bins (bin 1 within the deep cortical layers and bin 10 within the presumptive layer 1) within this rectangle, ImageJ, including the NeuronJ plugin, was used for counts and measurements (National Institutes of Health, Bethesda, USA).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) unless indicated otherwise. Continuous behavioral data were analyzed using repeated measures analysis of variance (ANOVA). In case of significant violations of sphericity or error variances, the Greenhouse-Geisser adjustment was applied and *df were* adjusted. Significant ANOVA results were followed by post hoc independent T-tests, in order to identify the specific time points with the largest effect of EFV. Differences in non-continuous data were analyzed by Pearson's Chi-square (or Fisher's exact). Immunohostochemical data were analyzed using one-way ANOVA. We observed extreme outliers in the acoustic startle/PPI test and removed these according to Tukey's principles. Cumulative behavioral development was defined using area-under-the-curves (AUC) for continuous data (righting reflex, negative geotaxis, bar holding) and sum scores for non-continuous data (swimming performance). Spearman's correlations were used to correlate behavioral development with immunohistochemistry results. Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism3 software (California corporation). All tests were performed using a two-tailed hypothesis, with significance set at 0.05. The Benjamini-Hochberg correction for multiple comparisons was applied to behavioral post hoc analyses - reported p-values represent raw p-values that are significant using FDR = 0.1.

Results

Pregnancy outcomes and maternal care

On GD 4, 1.5 hours following the administration of EFV, mean (± SEM) EFV plasma levels in EFV-treated dams (n = 4) were 0.28 ± 0.13 mg/l. Gestational length did not differ between EFV (22.0 ± 0.0 days) and controls

(22.3 ± 0.2 days, $p = 0.69$). No apparent anatomical abnormalities were seen in the EFV-exposed offspring. Furthermore, there were no significant differences in the number of pups per litter (EFV 10.8 ± 0.7 vs. controls 8.8 ± 1.3, $p = 0.34$), nor differences in the survival rates for pups at weaning. Finally, the mean (± SEM) percentage of female pups per litter did not differ between groups (EFV 42 ± 3% vs. controls 41 ± 6%, $p = 0.89$; Supplemental Table S1). To examine effects of EFV on dam-pup interactions, maternal behavior was scored from PND2 until PND9 and did not differ between groups ($p > 0.10$ for main effects of EFV and interaction between time and EFV; Fig. 2a).

General development

Fig. 2b shows the body weights of EFV- (n = 24) and vehicle-exposed (n = 19) rats during early life (PND2-PND21), adolescence (PND34) and adulthood (PND70). Body weight increased with age ($F_{(1.2,49.3)} = 6209.51$, $p < 0.0001$). Perinatal EFV exposure was associated with reduced weight gain (main effect EFV: $F_{(1,41)} = 29.09$, $p < 0.0001$, time x EFV interaction effect: $F_{(1.2,49.3)} = 7.99$, $p = 0.0045$). Body weight was significantly reduced in EFV-exposed rats for all testing days (post-hoc T-tests, $p < 0.010$). Eye opening was scored daily from PND13-PND18. On PND 17, EFV-exposed rats showed a delay in eye-opening ($p = 0.0027$, Fisher's exact; Fig. 2c).

Reflex and motor development

The time until the rats were able to roll from back to the front (righting reflex) reduced with age ($F_{(3.8,158.4)} = 20.09$, $p < 0.0001$; Fig. 2d). There were no main or interaction effects of EFV on righting time. In the negative geotaxis test, the time until turning decreased with age ($F_{(5.0,207.6)} = 127.04$, $p < 0.0001$; Fig. 2e). Rats perinatally exposed to EFV showed a developmental delay for negative geotaxis (main effect $F_{(1,41)} = 13.59$, $p = 0.00066$; time x EFV interaction $F_{(5.1,207.6)} = 0.1.17$, $p = 0.32$). Post-hoc analyses showed a trend for longer turning times in EFV-exposed animals on PND11 and PND12 (post-hoc T-tests, $p = 0.075$). We observed an improvement of performance in the bar holding test over time ($F_{(7.5,306.6)} = 35.02$, $p < 0.0001$; Fig. 2f). We did not see any main effect of EFV on bar holding ($F_{(1,41)} = 1.17$, $p = 0.285$), but we observed a trend in time x EFV interaction ($F_{(7.5,306.6)} = 1.81$, $p = 0.079$), with significant differences between groups on PND20 (post-hoc T-test, $p = 0.0048$). In the swimming test, EFV-exposed rats scored

significantly lower than their controls on PND14 ($p = 0.00046$, Fisher's exact; Fig. 2g). Finally, startle reflexes were assessed. The amplitudes of the startle response increased over time ($F_{(1.5,57.1)} = 33.32$, $p < 0.001$; Fig. 3a), with a main effect of EFV ($F_{(1,37)} = 5.65$, $p = 0.023$), and a trend for time x EFV interaction ($F_{(1.5,57.1)} = 2.72$, $p = 0.087$). Post-hoc analyses showed trends for lower startle responses in the EFV group in early life (PND21, post-hoc T-test, $p = 0.066$) and adolescence (PND35, post-hoc T-test, $p = 0.051$), but not in adulthood (PND70, post-hoc T-test, $p = 0.95$). Taken together, these data demonstrate that EFV-exposure affected early reflex and motor development, which normalized in later life.

Development of sensorimotor gating and anxiety- and depressive-like behavior

For all prepulses, the effects of PPI increased over time ($F_{(2,64)} = 58.44$, $p < 0.001$ for 3 dB, $F_{(2,70)} = 33.21$, $p < 0.001$ for 5 dB, and $F_{(1.18,42.4)} = 8.57$, $p = 0.004$ for 10 dB) We did not observe any main or interaction effects of EFV on PPI, regardless of the prepulse strength (Fig. 3b,c,d). We found no group differences in elevated plus maze open arm time ($F_{(1,41)} = 0.01$, $p = 0.92$; Fig. 3e). The number of open arm entries ($F_{(1,41)} = 0.11$, $p = 0.74$) and closed arm entries ($F_{(1,41)} = 0.01$, $p = 0.92$) also did not differ between control- and EFV-exposed rats (Fig. 3f,g), suggesting that EFV did not affect anxiety. Finally, no differences were found in the total distance moved ($F_{(1,41)} = 0.86$, $p = 0.361$; Fig. 3h), indicating that EFV had also no effect on exploratory locomotion. While there were no differences between the groups in the total duration of immobility ($F_{(1,39)} = 0.64$, $p = 0.48$), or the total number of immobility counts ($F_{(1,39)} = 0.598$, $p = 0.44$), there was a significant difference in latency to the first episode of immobility ($F_{(1,39)} = 9.88$, $p = 0.003$), with EFV-exposed rats exhibiting reduced latencies (Fig. 3i-k).

Motor cortex cytoarchitecture

To investigate whether perinatal EFV exposure affected the architecture of the primary motor cortex (M1), we assessed the M1 cell composition in randomly selected brains from adult animals (PND73-75; Fig. 4a). We observed a reduction in the total number of $DAPI^+$ cells in the M1 of the EFV-exposed compared to the control group ($p = 0.045$; Fig. 4a, b, c and Supplemental Figure S1b). This decrease was present in the superficial layers (bin8, $p = 0.014$; bin9, $p = 0.00030$) and to a lesser extent in deep layers (bin6,

p = 0.0096). Groups did not differ, however, in M1 cortical thickness (p= 0.37; Supplemental Figure S1g). To assess whether the observed reduction in cells was neuron-specific, we immunostained the M1 for the neuronal nuclei marker NeuN (Fig. 4a, d, e). We found a significant reduction of $NeuN^+$ neurons in the M1 of the EFV-exposed group ($p < 0.0001$; Fig. 4d and Supplemental Figure S1c), which was present in all layers (bin1-bin9, $p < 0.05$; Fig. 4e). Next, we focused on cell apoptotic features of perinatal EFV exposure in the M1 (Fig. 4a, f, g). We observed a significant increase of $Casp3^+$ cells in the M1 of EFV-exposed animals ($p = 0.023$; Fig. 4f and Supplemental Figure S1d), that was apparent in deep (bin1, $p = 0.0087$; bin3, $p = 0.0059$; bin5, $p = 0.049$ and bin6, $p = 0.0071$) and superficial layers of the M1 (bin7, $p = 0.023$ and bin9, $p = 0.041$; Fig. 4g) of the M1. The number of $NeuN^+$ neurons positive for Casp3 was higher in the EFV-exposed group compared to the control group ($p = 0.037$; Fig. 4h, I and Supplemental Figure S1e, f). In addition, we noticed a small population of $Casp3^+$ cells that were astrocytes (positive for GFAP) (Supplemental Figure S1a). When comparing nuclear size (using NeuN), we observed significantly reduced nuclear sizes in superficial layers of the EFV-exposed group (bin8, $p = 0.0054$; bin9, $p = 0.0080$; Fig. 4j, k), suggesting a possible difference in apoptotic state. Together, these data suggest (neuronal) cell loss in M1 cortical areas which persists into adulthood in perinatally EFV-exposed animals.

Given the serotonergic pharmacological profile of EFV, we hypothesized that early EFV exposure could have long-term effects on the serotonergic system and potentially indirectly on other neurotransmitter systems such as the catecholaminergic system[39]. To test this hypothesis, we immunostained M1 cortical slices for 5-HT and TH (Fig. 5a, b) and found that perinatal EFV exposure was associated with increased $5\text{-}HT^+$ fiber length ($p = 0.028$; Fig. 5c, e) in deep and superficial layers of the M1 (bin2, $p = 0.034$; bin6, $p = 0.040$; Fig. 5c). We found no differences in TH^+ fiber length ($p = 0.19$; Fig. 5d, e), suggesting that perinatal exposure to EFV influences the development and maintenance of the serotonergic, but not the catecholaminergic, system.

Correlations between reflex and motor development and M1 cytoarchitecture

Swimming performance correlated positively with the number of cells ($DAPI^+$; $r_s = 0.631$, $p = 0.009$, Supplemental Figure S2) and post-mitotic neurons ($NeuN^+$; $r_s = 0.642$, $p = 0.007$) in the M1. In addition, we found a

significant positive correlation between negative geotaxis and 5-HT$^+$ fiber length (r_s = 0.700, p = 0.036) and a trend between negative geotaxis and the number of DAPI$^+$ cells in M1 (r_s = -0.492, p = 0.053). These results suggest that the M1 of animals that performed better on the motor-related tests contained more DAPI$^+$ cells, more neurons and shorter 5-HT fibers.

Discussion

In this study we demonstrate that perinatal exposure to EFV in rats results in a transient delay in reflex and motor development and in persistent changes in M1 cytoarchitecture. Since maternal care was not affected by EFV treatment, the behavioral changes most likely arise from direct EFV brain toxicity. Correspondingly, EFV-exposure caused persistent structural changes in motor cortical layers, reflected by a reduction in the number of mature neurons. The increased number of Casp3$^+$ neurons points to neuronal cell death as the underlying cause. Finally, we found increased serotonergic, but not catecholaminergic, innervation of the M1 in EFV-exposed rats compared to controls, indicating that EFV, either directly or indirectly, affects the serotonergic system.

At the outset of our study, no studies had been published on long-term neurodevelopmental effects of EFV. Given EFV' ability to interfere with the brain serotonergic system,[12-14] we focused on behavioral domains known to be affected by genetic or pharmacological inactivation of the 5-HTT: reflex development[22, 40], motor performance[21, 25], and emotion[24, 41]. We found that perinatal EFV exposure resulted in delayed reflex development, reflected by increased latencies in the negative geotaxis test, and reduced startle responses. The reduced startle responses may indicate decreased capability of these rats to react to new auditory stimuli, but also point to a reduced ability to initiate motor responses. Correspondingly, EFV-exposed rats performed poorer in the swimming and bar holding tests. Adequate swimming requires the smooth integrative organization of multiple reflexes, including vestibular reflexes and extensor-flexor reflexes, which progressively develop during the postnatal phase of CNS maturation[34]. The delayed development of swimming ability in EFV-exposed rats suggests that early EFV exposure might modify these integrated neuromuscular mechanisms. The fact that bar holding performance was reduced as well indicates that muscle strength may have contributed to reduced swimming performance. We observed no group differences in PPI, the elevated plus maze test, or behavioral despair in the forced swim test. We did, however, observe shorter latencies to immobility in the forced swim test,

possibly reflecting motor-related problems. Taken together, these results suggest that perinatal exposure to EFV affects reflex and motor development, but not emotional behavior. The results are in line with recent findings showing that, compared to HIV-unexposed infants, HIV-uninfected infants perinatally exposed to EFV (aged 12-14 months) are at an increased risk for motor delay (particularly those born prematurely)[6].

Alterations in care provided by the mother can potentially mediate effects of EFV on motor development. As one-third of EFV-treated adults report CNS symptoms[10, 42], and EFV-treated adult rats show anxiety and depressive-like behavior[14, 43], dams treated with EFV may have provided lower-quality care of pups than mothers treated with vehicle. For this reason, we measured maternal care and did not find significant differences between EFV- and vehicle-treated dams. Hence, the observed behavioral and morphological neurodevelopmental changes in EFV-exposed rats likely reflect (in)direct drug toxicity, rather than indirect effects mediated by altered maternal care.

To elucidate to what extent perinatal drug toxicity effects could explain the observed delay in reflex- and motor development, we investigated the cytoarchitecture of the motor cortex (region M1) in adult rats. In the M1 of perinatally EFV-exposed rats, we observed a marked decrease in total cell numbers, including mature neurons, and an increase in the number of neurons that expressed the apoptotic marker Casp3. These findings indicate that perinatal EFV causes cell death later in life, leading to a partial loss of neurons. The positive correlation between motor development and number of cells suggests that the delayed motor function is related to reduced cell number in the M1. The EFV-induced loss of cells may be attributed to EFV' (in)direct ability to interfere with cellular metabolism. For example, EFV has been shown to decrease neuronal ATP storage, leading to increased levels of reactive oxygen species and cell death[44, 45].

Alternatively, perinatal EFV exposure could indirectly lead to neurodevelopmental changes and cell death, by targeting the serotonergic system. We therefore determined whether the observed neuronal loss and cell death were related to changes in the serotonergic system. We measured the length of 5-HT$^+$ and TH$^+$ fibers in the M1 and observed an increased length of 5-HT$^+$, but not TH$^+$, fibers, which correlated with reduced performance in the negative geotaxis test. This result resembles the case of perinatal SSRI exposure, which also results in increased cortical serotonergic innervation in later life[19, 46]. 5-HT is one of the earliest neurotransmitter in the brain and performs neurotrophic functions during early development[47]. Hence, any

interference with the serotonergic system by perinatal drug exposure can have profound long-lasting indirect effects on neurodevelopmental events. The 5-HT_2, 5-HT_3, and 5-HT_6 receptors are involved in cell division[48], differentiation[49], survival[50], and neuronal migration pathways[47]. EFV, as a 5-HT receptor (ant)agonist[13], could directly interfere with these processes, consequently leading to neuronal cell death[45]. Elucidation of the exact molecular and cellular consequences at various developmental time points after perinatal EFV exposure in cortical and other brain areas requires further research.

We observed no signs of EFV-related pregnancy complications or teratogenicity, which is in line with findings in a recent meta-analyses including 2026 first-trimester EFV-exposures[5]. Still, EFV-exposed rats showed delayed maturation (delayed eyelid opening) and reduced body weights throughout life. Reduced birth weights have been reported in EFV-exposed infants, although most studies in humans report comparable birth weights with infants exposed to other ART[4, 51, 52]. Interpretation of these data is difficult as drug-related effects cannot easily be isolated from other HIV-related factors. Interestingly, the reduced body weight finding is in line with animal and human data derived from other early-life 5-HT stimulation models[24, 25, 53]. There are several ways in which EFV exposure might reduce body weight: via 5-HT, which through hypothalamic 5-HT receptors can interfere with the central regulation of eating behavior[54], or via 5-HT-independent mechanisms such as increased metabolism and altered adipocyte differentiation[55].

In contrast to other behavioral EFV studies[14, 43], we measured plasma levels of EFV. Using a 100 mg/kg daily dose, we produced EFV plasma levels that were detectable, yet below the therapeutic range in humans (1.0-4.0 mg/L)[56]. One may argue that our experiment was, therefore, not representative for the human situation. However, it is known that the main neurotoxic metabolite of efavirenz approximates human exposure, despite apparent relatively low efavirenz plasma levels[55]. In addition, even at relatively low concentrations, EFV has been shown to extensively accumulate in brain tissue in rats[57]. Lastly, at similar doses in rats, efavirenz has been shown to induce neurotoxic changes[12]. We, therefore, postulate that our experimental set-up was sufficiently valid to induce the desired effects.

The major strength of using a rat model is that potential HIV-related and socioeconomic confounders of adverse neurodevelopmental effects of EFV in humans (e.g. parental loss; co-medication) can be eliminated. As EFV did not affect maternal care, developmental effects through care provided by

the mother can be excluded. Our study has also limitations. We performed multiple behavioral tests with the same animals, which could have influenced test outcomes. We expect this carry-over effect to be limited, as tests were generally not stressful. Furthermore, molecular and cellular experiments involved adult rats, while behavior was measured mostly during early life. Thus, while we observed correlations between motor performance and neuronal loss in M1, we cannot draw conclusions on the timeline of events exerted by perinatal EFV exposure eventually leading to cell loss. Finally, to provide a causal relationship between behavioral and molecular changes observed, an intervention (e.g. co-administration of 5-HT_{2A} receptor antagonist during efavirenz exposure) would be required.

There is a clear indication of HIV treatment during pregnancy as it not only protects the (unborn) child, but also benefits the mother's health. The vast majority of children exposed to EFV during pregnancy will be HIV-uninfected with normal life expectancies. However, this outcome may come at a cost. As we demonstrate, perinatal exposure to EFV in rats leads to a delay in reflex and motor development and a long-lasting loss of neurons in the motor cortex. Thus, EFV could affect the development of children who may already be experiencing multiple adverse conditions (such as having a mother living with HIV). Our findings underline the need for long-term clinical studies in children that are perinatally exposed to EFV, as well as more detailed studies on the underlying neurodevelopmental mechanisms.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data deposition

Datasets used in this manuscript will be shared upon request to the corresponding authors.

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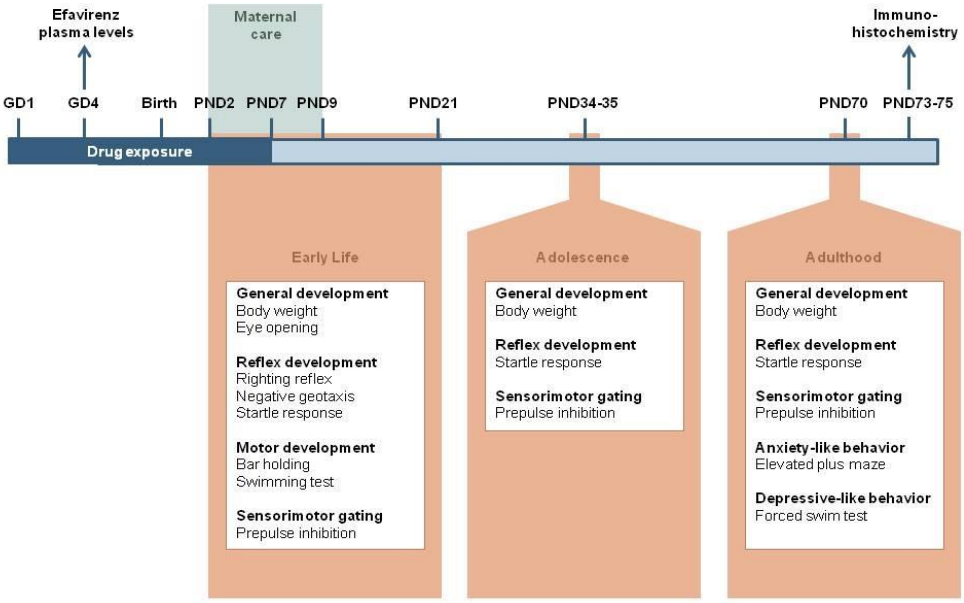


Fig. 1. Experimental timeline. Pregnant dams were treated with efavirenz (n = 4) or vehicle (n = 4) from GD1 until PND7. At GD4, plasma levels of efavirenz were determined 1.5 hours post administration. Developmental milestones and behavior were assessed during the first three weeks of life (PND2-21), adolescence (PND34-35) and adulthood (PND69-70). Maternal care was monitored from PND2 until PND9. After completion of the last experiments, rats (PND73-75) were sacrificed and their brains were removed for immunohistochemistry analyses.

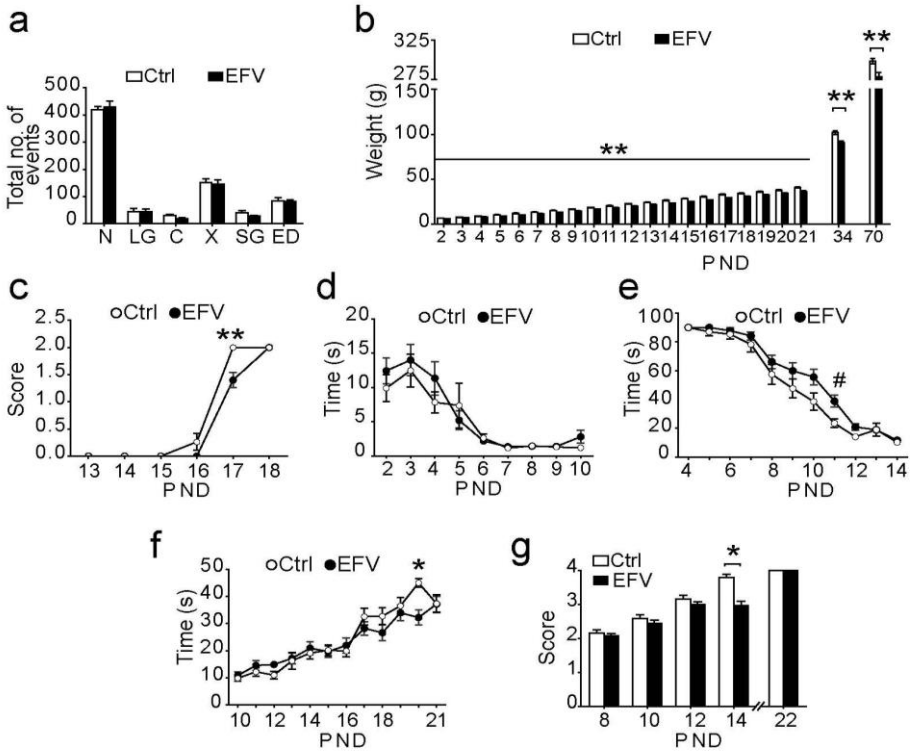


Fig. 2. Perinatal exposure to EFV significantly affects bodyweight, eye opening, reflex and motor development, without effects on maternal care. EFV: efavirenz; Ctrl: vehicle; PND: postnatal day. (**a**) No effect of EFV on maternal behaviors N: nursing; LG: licking/grooming; C: contact; X: out of nest; SG: self-grooming; ED: eating/drinking (n = 4 litters per group, PND2-9). (**b, c**) Effects of perinatal EFV on general development (**b**) Main effect of EFV on bodyweight, with EFV-exposed rats showing reduced body weight (**c**) EFV affects eye opening (PND17) scored as (0) both eyes closed; (1) one eye open; (2) two eyes open (**d, e**). Effect of perinatal EFV on reflex development (**d**) No effect of EFV on righting time (s) in the righting reflex test (**e**) Main effect of EFV on negative geotaxis test, with EFV-exposed rats exhibiting longer latencies (s) until turning 180˚ (**f, g**) Effects of perinatal EFV on motor development (**f**) No main effect of EFV on the latency (s) to fall off the bar in the bar holding test, with a trend in EFV x time interaction (**g**) EFV affects swimming performance (PND14), scored as (1) entire head underwater, (2) nose underwater, ears partially underwater with the back of head above water level, (3) nose above water, ears partially underwater, (4) entire head above water (PND8, 10, 12, 14, 22). EFV n = 24, Ctrl n = 19. Data represent mean (± SEM), $^{\#}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, analyzed using repeated measures ANOVA (a, b, d, e, f) or Fisher's exact (c, g).

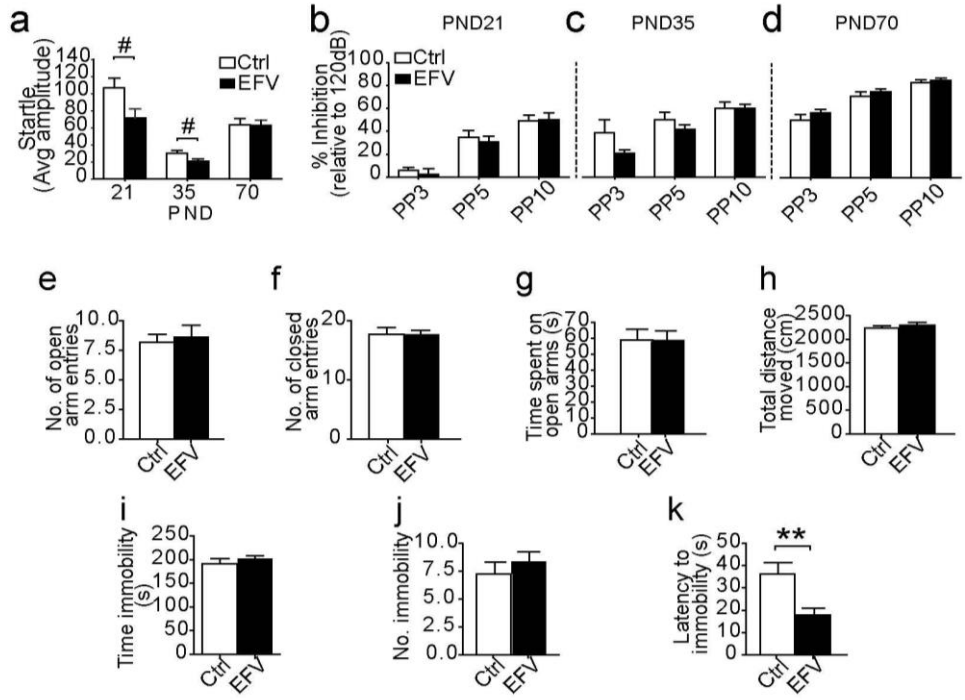


Fig. 3. Perinatal EFV exposure affects the startle reflex and latency to immobility in the forced swim test, but has no effects on prepulse inhibition, or anxiety- and depressive-like behavior. EFV: efavirenz; Ctrl: vehicle; PND: postnatal day; PP: prepulse. (**a-d**) Effect of perinatal EFV on acoustic startle reflexes and prepulse inhibition (PPI). (**a**) Main effect of EFV on average startle amplitude development, with EFV-exposed rats exhibiting shorter startle amplitudes. No effect of EFV on PPI on (**b**) PND21, (**c**) PND35, and (**d**) PND70. PPI was calculated as: 100-(Average of all startle amplitudes on prepulse trial/startle amplitude on startle trial) x 100%. Three different prepulses were tested: 3 dB (PP3), 5 dB (PP5) or 10 dB (PP10) above background noise. (**e-h**) No effect of perinatal EFV on anxiety-like behavior in the elevated plus maze. (**e**) Number of open arm entries, (**f**) Number of closed arm entries, (**g**) Time (s) spent in open arms, and (**h**) Total distance (cm) moved. (**i-k**) Effects of perinatal EFV on depressive-like behavior in the forced swim test (**i**) No effect of EFV on the time (s) spent immobile, or (**j**) the number of immobility times. (**k**) Main effect of EFV on the latency (s) to immobility, with EFV-exposed rats exhibiting shorter latencies. EFV n = 22-24 and Ctrl n = 17-19. Data represent mean (± SEM), $^{\#}p < 0.10$, $^{**}p < 0.01$, analyzed using repeated measures ANOVA (a-d) or One-way ANOVA (e-k).

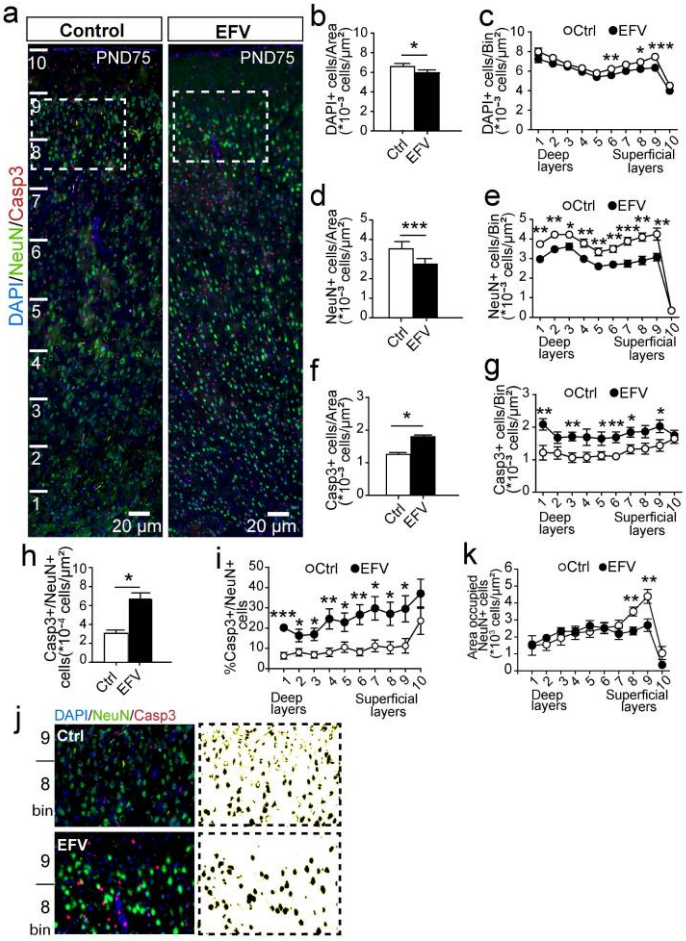


Fig. 4. Perinatal exposure of EFV increases apoptosis in neurons of the motor cortex (M1) **(a-k).** EFV: efavirenz; Ctrl: vehicle **(a)** Immunostaining for DAPI, mature neurons (NeuN) and apoptosis (Caspase-3; Casp3) in the primary motor cortex (M1) of EFV- (n = 8) and Ctrl-exposed (n = 8) animals. The boxed areas are enlarged in **(j)**. **(b, c)** Significant decrease of the total number of $DAPI^+$ cells in the superficial cortical layer of the EFV–exposed group. **(d, e)** Quantification of mature neurons ($NeuN^+$) showing a significant decrease in $NeuN^+$ cells in deep and superficial layers in the EFV-exposed group. **(f, g)** Quantitative results showing a significant increase in $Casp3^+$ cells in deep and superficial layers of the M1 of EFV-exposed animals. **(h-i)** A significant increase in $Casp3^+/Neun^+$ cells in the EFV group compared to controls. **(k)** Significant differences in the area occupied by $NeuN^+$ nuclei in 2 bins of superficial layers between EFV-treated and control animals. Data show mean (± SEM) number of cells, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA.

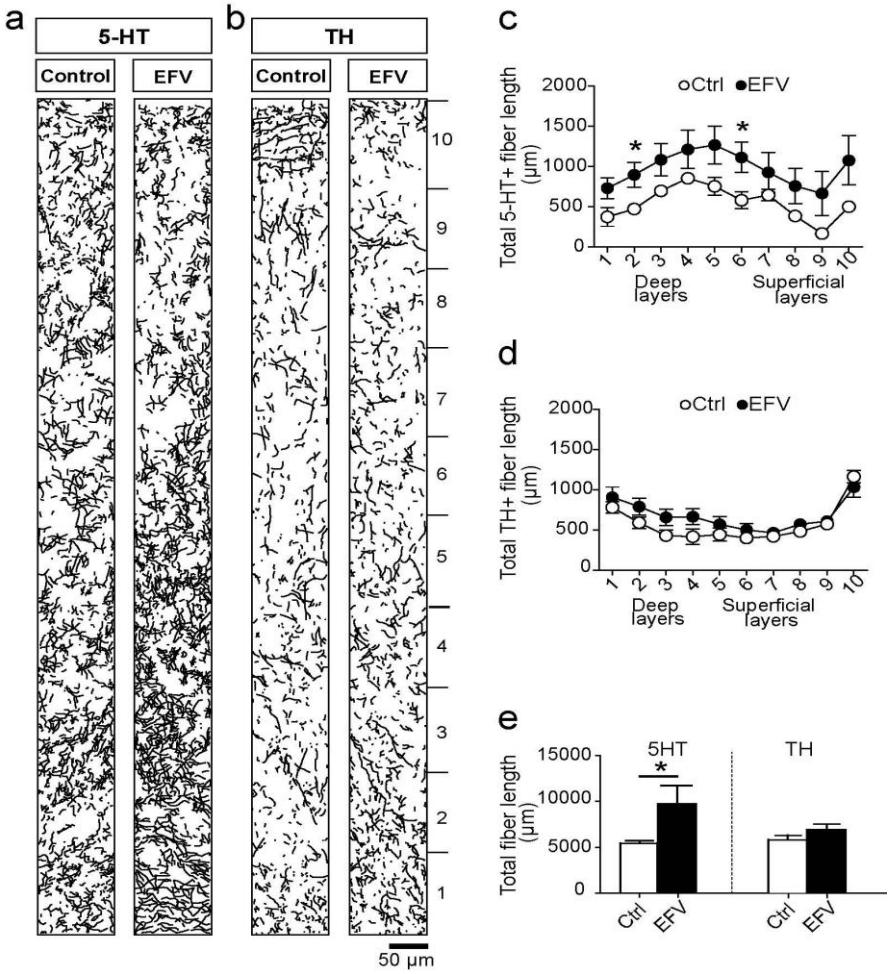
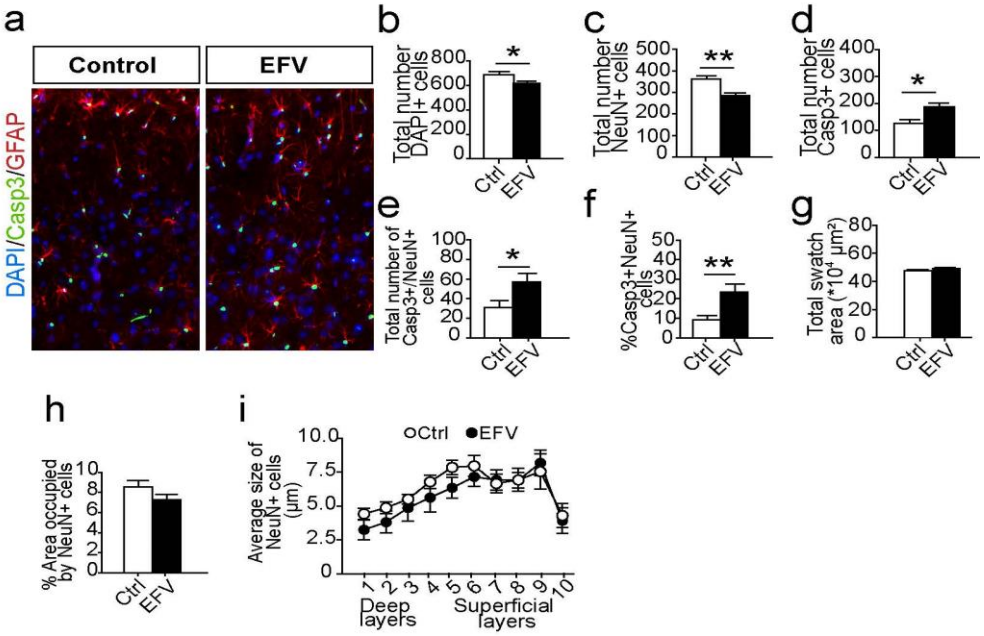


Fig. 5. Perinatal exposure to EFV affects serotonergic (but not catecholaminergic) innervation of the M1. EFV: efavirenz; Ctrl: vehicle; 5-HT: serotonin; TH: tyrosine hydroxylase. (**a, b**) Camera lucida drawings of immunostaining for (**a**) 5-HT$^+$ and (**b**) TH$^+$ fibers in deep and superficial layers of the M1 in EFV-exposed (n = 5-7) and Ctrl-exposed brains (n = 5-7). (**c**) Significant increase in 5-HT$^+$ fiber length in bin 2 and 6 in the EFV group (**d, e**) Significant increase in 5-HT$^+$ fiber length, but no difference in TH$^+$ fiber length in the total M1 area of the EFV-exposed group. Data show mean (± SEM) number of fibers, *$p < 0.05$ analyzed using One-way ANOVA.

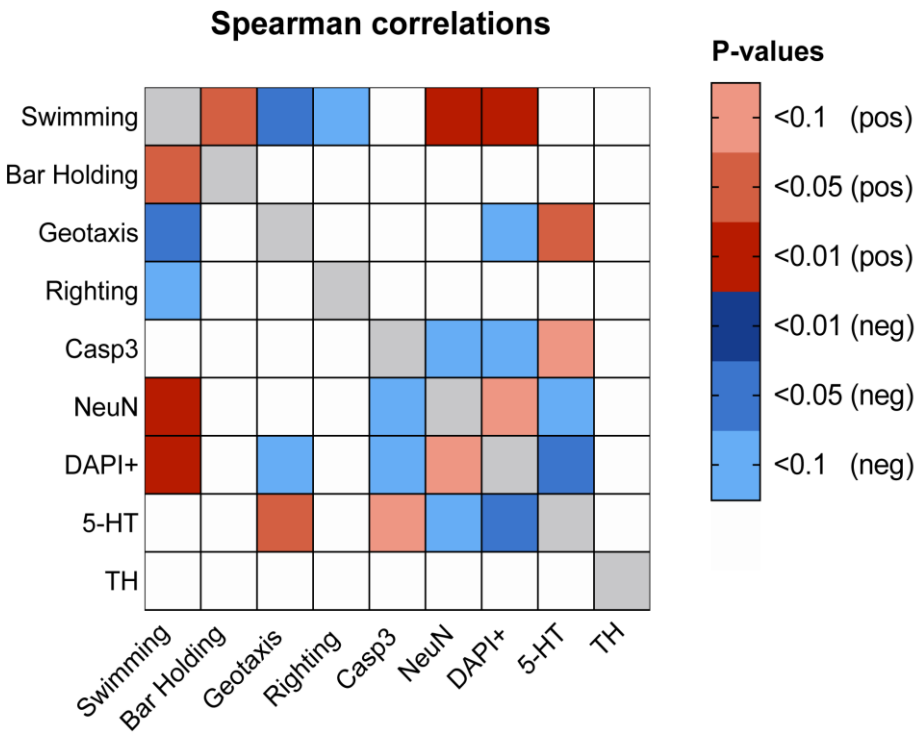
Supplementary Table 1. General characteristics litters

	Control (n = 4)	**EFV (n = 4)**	**P-value**
Gestational length, days	22 (0.0)	22 (0.2)	0.686
No. of pups per litter	8.8 (1.3)	10.8 (0.7)	0.343
No. of still births	0 (0.0)	0 (0.0)	-
No. of neonatal deaths	0.0 (0.0)	0.5 (0.3)	0.343
Gender pups, % female	41 (12)	42 (7)	0.886
EFV plasma level (mg/L)	-	0.28 (0.13)	-

Data are depicted as mean (± SEM)



Supplementary Fig. 1. Perinatal exposure of EFV increases apoptosis in several types of motor cortex cells including astrocytes (a-f) but has no effect on motor cortical thickness. EFV: efavirenz; Ctrl: vehicle (**a**) Swatch of immunostaining showing colocalization with cleaved-Caspase-3 (Casp3) and GFAP, an astrocyte marker, in the motor cortex of EFV- (n = 8) and Ctrl-exposed (n = 8) animals. (**b, c, d**) Significant decrease in the total number of cells (**b**), total number of mature neurons (**c**) and total number of cells that are $Casp3^+$ (**d**) in the EFV-exposed group compared with the Ctrl-exposed group. (**e**) Significant increase in the total number of $Casp3^+$ neurons. (**f**) Increased percentage of neurons that are $Casp3^+$ in the EFV-exposure group. (**g**) No differences in the total length of a cortical swatch (**h, i**) No differences in the percentage of area occupied by $NeuN^+$ cells and average size of $NeuN^+$ cells in EFV group compared to the control group. Data show average ± SEM. One-Way ANOVA, *$p < 0.05$, **$p < 0.01$.



Supplementary Fig. 2. Spearman correlations between behavioral development and immunohistochemistry data of control (n = 8) and efavirenz-exposed (n = 8) animals. Cumulative behavioral development was defined using area-under-the-curve for righting reflex (PND2-10), negative geotaxis (PND4-14) and bar holding (PND10-21) and total sum score for swimming performance (PND 8, 10, 12, 14, 22). Immunohistochemistry data reflect the total number of $DAPI^+$ cells, mature neurons ($NeuN^+$), apoptotic cells (cleaved-Caspase-3 (Casp3)), total serotonin ($5\text{-}HT^+$) fiber length, and total TH^+ fiber length in the primary motor cortex (M1).

6

Perinatal exposure of rats to the HIV drug efavirenz affects medial prefrontal cortex cytoarchitecture

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In preparation for submittance

Abstract

Efavirenz (EFV) is used for antiretroviral treatment of HIV infection, and successfully inhibits viral replication and mother-to-child transmission of HIV during pregnancy and childbirth. Unfortunately, the drug induces side effects regarding cognitive functioning, including neuropsychiatric symptoms such as anxiety and depressed mood. EFV acts on, among others, the receptors and transporter of serotonin that are expressed throughout the developing brain. Yet, how perinatal EFV exposure affects brain cytoarchitecture is still unclear. Here, we exposed pregnant and lactating rats to EFV, and examined in the prefrontal cortex (PFC) of their offspring the effects of the maternal EFV exposure on cortical architecture. We observed a significant decrease in the number of cells, mainly mature neurons, in the infra/prelimbic and cingulate cortices of adult offspring. Futhermore, an altered cortical cytoarchitecture characterized by a significant reduction in deep-layer $Tbr1^+$ and superficial-layer $Cux1^+$ cells was found. We also observed a sharp increase in programmed cell death as we identified a significantly higher number of cleaved-Caspase-3^+ cells. In addition, the serotonergic and dopaminergic innervations of the PFC subdomains were increased. Thus, the perinatal exposure to EFV provoked in the mPFC of adult offspring cell death, significantly altered cytoarchitecture, and disturbances in serotonergic and dopaminergic innervations. Our results are important in light of EFV treatment of HIV-positive pregnant women, and its effect on brain development and cognitive behavior.

Keywords: Prefrontal Cortex Development, PFC, Corticogenesis, Neurodevelopment, Efavirenz, Antiretroviral Therapy, Serotonin, Side Effect.

Introduction

For the treatment of HIV infection, antiretroviral therapy is used, reducing morbidity and mortality significantly by decreasing retroviral replication[1]. International guidelines for HIV treatment recommend the use of two first-line nucleoside reverse transcriptase inhibitors and a third drug, which should be either an enhanced protease inhibitor, an integrase strand transfer inhibitor, or a non-nucleoside reverse transcriptase inhibitor. Efavirenz (EFV) is a drug that falls within the last category[2,3]. EFV effectively reduces viral replication, displays a low degree of interaction with the other antiretroviral drugs, is low in costs, requires single daily dosing and has moderate side effects which facilitates adherence to the treatment. Because of these characteristics, EFV is advised to be used by pregnant women to prevent the transfer of HIV from mother to child, decreasing the chance of infection to 5%[1,4,5,6].

During the prenatal period, EFV crosses the placenta freely[7], reaching the fetal blood and is able to cross the blood-brain barrier[8] with potential effects on the developing brain. In the postnatal period, EFV is found in breast milk[9,10], indirectly reaching the fetal blood and brain, to possibly affect brain development as well. The potential teratogenic effects of EFV are still not well described[2,11]. Prematurity, low birth weight and anomalies in the development of the neural tube have been demonstrated when EFV was used by the mother[12,13,14]. Preliminary data from a pioneer study conducted in children with fetal EFV exposure showed that these children exhibit poorer neurodevelopmental and social-emotional outcomes than children with fetal exposure to non-EFV-based antiretroviral regimens[15]. In addition, exposure of pregnant cynomolgus monkeys to EFV at the onset of gestation caused anencephaly and palatine cleft in 15% of the animals exposed at therapeutic EFV doses[1,16]. In rats, perinatal exposure to EFV led in the offspring to a delay in motor development as well as disturbances within the cytoarchitecture of the motor cortex[17].

EFV can bind as an agonist or antagonist to vesicular monoamine transporters, dopamine and serotonin (5-HT) transporters, and glutamate, GABA, muscarinic, and the 5-HT receptors 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_6[18,19]. EFV's effect on 5-HT receptors and transporter is of particular relevance for brain development, since 5-HT functions as a neurotrophic factor responsible for controlling the events of proliferation, migration, differentiation and cell death during fetal and early postnatal brain development[20-24]. Before the serotonergic raphe nucleus in the embryonic

brain starts to produce 5-HT around embryonic day (E)10.5, the embryo receives 5-HT from the placenta[25,26]. In rats, the dorsal and medial raphe nucleus, located in the brain stem, start to develop and send their fibers to the forebrain which at around E16.5 reach the medial prefrontal cortex (mPFC). Once the serotonergic fibers are able to supply 5-HT to all brain regions (around E18.5), the placenta stops the production of 5-HT[25,26]. It is known that selective pharmacological or genetic interference with components of the 5-HT signaling pathway in pregnant rodents leads to a variety of behavioural changes, including increased anxiety, depression-related behavior in the context of stress, decreased behavioral flexibility and deficits in social behavior[27,28,29]. Furthermore, a prenatal pharmacological/genetic block of the 5-HT transporter (5-HTT) has been associated with an increase in 5-HT and dopamine innervation in the mPFC of embryos and pups, a disorganization of the cytoarchitecture of cortical layers[30,24] as well as a down/up-regulation of numerous serotonergic receptors in various cortical areas including the mPFC[31,32,33]. Thus, a critical question is whether perinatal EFV exposure affects the cytoarchitecture of the mPFC as well.

Here, our objective was to explore the effects of EFV-exposure throughout the gestational period and during the first postnatal week on the infralimbic, prelimbic and cingulate cortex subareas of the rat mPFC, and to determine if any changes were maintained during adult life. We found that perinatal EFV exposure decreased the number of cells, in particular mature neurons, in all mPFC subareas studied and increased the expression of the apoptotic marker cleaved-Caspase-3. We furthermore observed differences in the expression of layer markers and increased serotonergic and dopaminergic innervations of the mPFC. Because in mPFC subareas EFV causes significant disturbances in cortical cytoarchitecture, our results are important when considering EFV-treatment of HIV-positive women during their pregnancy and lactating period.

Material and methods

Animals

All experiments were approved by the Animal Experimentation Committee of the Radboud University Medical Center Nijmegen, The Netherlands (ref no. 2012-236) and carried out in accordance with the Directive of the Council of European Communities (2010/63/EU). Male and female nulliparous Wistar rats weighing 185-215g (Charles River, Cologne,

Germany) were acclimated and housed together. After detection of the vaginal plug, at the first gestational day (GD1), pregnant females were randomly housed in pairs in Macrolon® type 3 standard cages in temperature-controlled rooms (21°C ± 1°C) under a 12-hour standard light/dark cycle (lights on at 7:00 a.m.) with food and water available *ad libitum*.

Drug treatment

Pregnant rats were randomly assigned to daily treatment with either EFV or vehicle from GD1 until postnatal day 7 (PND7). The drug solution was prepared by diluting the oral suspension of EFV (Stocrin suspension 30 mg/ml, Merck Sharp & Dohme, Haarlem, The Netherlands) with distilled water. As a vehicle, we used a 1% cellulose suspension (Genfarma BV, Maarssen, The Netherlands), enriched with additives from the EFV solution, consisting of medium chain triglyceride oil (Newpharma, Liège, Belgium) and strawberry and mint flavors (Lecocq NV/SA, Zonhoven, Belgium). The EFV or vehicle was given blindly by oral gavage in a volume of 5 ml/kg. A dose of 100 mg/kg was used, based on unpublished pilot work and previous work demonstrating plasma levels within the human therapeutic range (1.0-4.0 mg/l)[2]. Plasma EFV levels were measured on the fourth gestational day (GD4) 90 minutes after drug administration in a blood sample obtained via the tail and collected in Microvette CB 300 tubes (containing EDTA, Sarstedt, Germany), and the sample was processed as described by Wijer et al.[36]. At PND70, male offspring were sacrificed, and their brains removed for immunohistochemical studies.

Immunohistochemistry

The animals received a single intraperitoneal (IP) injection of sodium pentobarbital (200 mg/kg), causing deep anesthesia. They were perfused transcardially with cold phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. Brains were rapidly removed and immersed in 4% PFA in PBS for 48 hours at 4°C. After 2 days, the brains were immersed in a 30% sucrose solution in PBS until they sunk, frozen and stored at -80°C. 16 µm sections were cut on a Microm Cryostat, mounted on Superfrost® Plus slides (Thermo Fisher Scientific, Waltham, MA, USA), air dried and stored desiccated at -20°C. After one hour in blocking buffer (BB, 1.7% normal goat serum (NGS), 1.7% normal donkey serum (NDS), normal horse serum (NHS), 0.1% lysine, 1% BSA, 1% glycine and 0.4% Triton X-

100), the cryosections were stained immunohistochemically using the following primary antibodies: mouse anti-Cux1 (1:300, Abcam, ab242194, Cambridge, United Kingdom), rabbit anti-Tbr1 (1:500, Abcam, ab31940, Cambridge, United Kingdom), mouse anti-NeuN (1:500, Merck Millipore, Bedford, MA, USA; MAB377), rabbit anti-cleaved-Caspase-3 (cl-Casp3, 1:500, Cell Signaling Technologies, Danvers, MA, USA, ASP175), rabbit anti-hydroxytryptamine (5-HT) (1:500, Sigma-Aldrich, Zwijndrecht, The Netherlands, S5545) and rabbit anti-tyrosine hydroxylase (TH) (1:500, Merck Millipore, Bedford, MA, USA; AB152), all diluted in blocking buffer. After overnight incubation, the slides were washed in PBS and incubated with the corresponding species-specific Alexa-conjugated secondary antibody (1:500, Thermo Fisher Scientific) in blocking buffer for 30 minutes at room temperature. After washing in PBS, the sections were contrasted with DAPI diluted in PBS (1:1000, Thermo Fisher Scientific) for 15 minutes, washed extensively in PBS and embedded in 90% glycerol in PBS. For visualization, a Leica DMI6000B Automated high-content fluorescence microscope was used.

Quantification and analysis

All analyses were performed in a blind fashion on at least six sequential coronal sections of each mPFC subarea (infralimbic, prelimbic and cingulate), which were scanned for cell and fiber quantification. The counting area in the mPFC was obtained using a rectangle of 0.1 mm wide extending from the ventricular zone to the marginal zone, using Photoshop CS6 (Adobe). This rectangle was subdivided into 10 equal bins, in which bin 1 represents the deepest layer and bin 10 the most superficial layer. For counting of the cells, Photoshop CS6 (Adobe) tools were used and fiber lengths were measured using ImageJ software, including the NeuronJ plugin (National Institutes of Health, Bethesda, USA). Data were statistically analyzed by one-way ANOVA ($\alpha = 5\%$) using GraphPad Prism 6/Excel data analysis toolkit and expressed as mean ± SEM.

Results

Perinatal EFV exposure affects the architecture of mPFC subareas

In a previous study, we found a clear effect of EFV on the maturation of the motor cortex and correlated behavior. However, it was unclear whether

this effect also occurs in other cortical structures that direct complex behavior[36]. In order to investigate the effect of perinatal EFV exposure on prefrontal development, the cytoarchitecture of various subdomains (infralimbic, prelimbic and cingulate) of adult (PND75) mPFC was studied. In rats perinatally exposed to EFV, we observed a significant reduction in the total number of $DAPI^+$ cells in the adult infralimbic ($p = 0.00065$; Fig. 1B and C), prelimbic ($p = 0.034$; Fig. 1B and D), and cingulate cortex ($p = 0.00085$; Fig. 1B and E) when compared to the control group. This cellular reduction was distributed over the cortical swatch of all subdomains, but most apparent in the deeper layers (Fig. 1F, G, H).

To search for the identity of this reduced number of cells in the mPFC, we quantified the number of cells positive for the neuronal marker NeuN in the mPFC subareas. We found a significant reduction in the total number of $NeuN^+$ cells per area in the deep as well as superficial cortical layers of the infralimbic (bin1, $p = 0.0071$; bin2, $p = 0.025$; Fig. 1B and I), prelimbic (bin1, $p = 0.0063$; bin2, $p = 0.012$; bin3, $p = 0.0060$; bin4, $p = 0.013$; bin5, $p = 0.016$; bin6, $p = 0.019$; Fig. 1B and J) and cingulate cortex (bin1, $p = 0.0022$; bin2, $p = 0.00091$; bin3, $p = 0.0018$; bin4, $p = 0.0016$; bin5, $p = 0.0011$; bin6, $p = 0.00016$; bin7, $p = 0.00014$; bin8, $p = 0.0060$; bin9, $p = 0.00044$; Fig. 1B and K) of animals perinatally exposed to EFV compared to the control group. Using neocortex laminar/identity markers, we detected a significantly lower number of cells immunoreactive for the deep layer marker Tbr1 in all subareas of the mPFC of the EFV-exposed rats compared to control animals (infralimbic, $p = 0.0019$; prelimbic, $p = 0.017$; cingulate cortex, $p = 0.014$; Fig. 2A-G; Supplemental Fig. 1A, B, C). In addition, we found a significantly lower number of cells positive for the superficial layer marker Cux1 in all cortical layers of the infralimbic ($p = 0.0083$; Fig. 3A, B, E; Supplemental Fig. 1D), prelimbic ($p = 0.0013$; Fig. 3A, C, F, Supplemental Fig. 1E), and cingulate cortex ($p = 0.0066$; Fig. 3A, D, G; Supplemental Fig. 1F) of animals exposed to EFV compared to controls. In a small population of cells in the upper and deep layers, we identified double-positive $Tbr1^+/Cux1^+$ cells[37] which showed a significant decrease in the deeper aspects of laminar distribution of mPFC prelimbic (bin2, $p = 0.033$; Fig. 4C, F; Supplemental Fig. 1H) and cingulate cortex (bin1, $p = 0.029$; bin2, $p = 0.030$; bin3, $p = 0.019$; bin4, $p = 0.030$; bin5, $p = 0.035$; Fig. D and G; Supplemental Fig. 1I) of the EFV-treated group compared to the controls. However, in the infralimbic cortex, a significant reduction of these double-labeled cells was found in not only deep but also in superficial layers (bin2, $p = 0.029$; bin5, $p = 0.048$; bin6, $p = 0.0085$; bin7, $p = 0.00081$; Fig. 4A, B, E; Supplemental

Fig. 1G). Even with cell loss, the cortical thickness between mPFC domains was not affected (Supplemental Fig. 1A-C). Together, the data suggest that perinatal EFV exposure alters the cytoarchitecture of mPFC subareas by reducing the number of neurons, along with an altered expression of deep and superficial cortical layer markers.

Perinatal EFV exposure results in neuron death in mPFC subareas

Programmed cell death is a natural and critical process during brain development[38]. Various apoptotic signaling pathways are mediated by activation of, among others, serotonergic signaling[38,39,40,41]. We observed a significant increase in cells positive for the apoptotic marker cleaved-Caspase-3 in the prelimbic (bin4, $p = 0.040$; bin5, $p = 0.049$; Fig. 5F) and cingulate cortex (bin1, $p = 0.011$; bin2, $p = 0.048$; bin4, $p = 0.015$; bin6, $p = 0.023$; bin8, $p = 0.0039$; bin9, $p = 0.039$; Fig. 5G) of adult (PND75) animals exposed to EFV when compared to control animals. No differences were identified in the infralimbic cortex ($p = 0.16$; Fig. 5E). Interestingly, not all cleaved-Caspase-3+ cells co-expressed NeuN (Fig. 5A). Only in the cingulate cortex, we found a significant difference in the total number of mature apoptotic neurons between the EFV and control groups ($p = 0.027$; Fig. 5D). We conclude that perinatal EFV exposure results in cellular apoptosis of mature neurons in mPFC subareas of adult animals.

Perinatal EFV exposure causes disturbances in serotonergic and dopaminergic innervations of mPFC subareas

Disturbances of serotonergic and dopaminergic innervations during the critical period of brain development are associated with various brain diseases[42,43]. Both neurotransmitters are able to control important neurodevelopmental processes such as proliferation, migration, differentiation, programmed cell death and synaptic organization during the embryonic period and adolescence, up to adulthood[44,39]. Therefore, we next quantified the lengths of the 5-HT^+ and TH^+ fibers in the infralimbic, prelimbic and cingulate cortex of adult (PND75) animals exposed to EFV perinatally. We found a significant increase in 5-HT^+ fiber lengths in the deep layers of the infralimbic (bin2, $p = 0.033$; bin3, $p = 0.021$; bin4, $p = 0.017$; Fig. 6A, B, E), prelimbic (bin1, $p = 0.016$; bin2, $p = 0.027$; bin3, $p = 0.037$; Fig. 6C and F) and cingulate cortex (bin2, $p = 0.031$; bin3, $p = 0.022$; Fig. 6D and G) of rats perinatally exposed to EFV compared to control animals. We

furthermore observed a significant increase in TH^+ fiber lengths in the deeper layers of the infralimbic (bin3, $p = 0.045$; Fig. 7A, B, G), prelimbic (bin6, $p = 0.039$; Fig. 7C, D, H) and cingulate cortex (bin1, $p = 0.010$; bin2, $p = 0.011$; bin5, $p = 0.0054$; bin6, $p = 0.018$; Fig. 7E, F, I). Together, the data suggest that changes in serotonergic and catecholaminergic systems occur in animals perinatally exposed to EFV and that these changes persist into adulthood.

Discussion

We have explored the question whether exposure of rats to EFV during the embryonic period and the first postnatal week results in adverse effects on mPFC development. Our results demonstrate that perinatal EFV exposure causes a significant decrease in the total number of cells, including mature neurons, in all subareas of the mPFC without affecting cortical thickness. Furthermore, EFV exposure results in disturbances in the cytoarchitecture as evidenced by a significantly reduced number of cells expressing Cux1 and Tbr1, markers of superficial and deep cortical layers, respectively. This may be due to the increased activation of apoptotic pathways as demonstrated by the increase of cleaved-Caspase-3^+ cells in all mPFC subdomains or an altered or delayed expression of the markers. In addition, we observed a significant increase in serotonergic and catecholaminergic innervations of the infralimbic, prelimbic and cingulate cortex of EFV-exposed rats. Together, the data suggest that perinatal EFV exposure causes prefrontal alterations at the cellular level in all subdomains of the mPFC and that these changes persist into adulthood.

The development of the PFC undergoes a number of stages. Following proliferation, cells progress to stages of neural migration, maturation, synapse formation, cell death, and network maturation[34,45-48]. All of these events are mediated by local neurotrophic factors and other external afferents, including serotoninergic and catecholaminergic ones[24]. It has been demonstrated that 5-HT, via various receptors present on different neural progenitor cells, glial cells and Cajal-Retzius cells, can regulate specific events during cortical development, including neuronal maturation, excitability and programmed cell death[20,21,22]. The effects of EFV on the immature brain may be broad, since this drug binds as an agonist or antagonist to a number of receptors and transporters of the 5-HT signaling pathway as well as of other neurotransmitter systems[19,18]. This interaction of EFV with different serotonergic receptors and transporter could be behind disturbances in neuronal migration and increased programmed neuronal death found by us.

Which together resulted in disorganized layers of the mPFC in the face of increased serotonergic and dopaminergic innervation. Recently, we found that rats exposed perinatally to EFV showed an increase in serotonergic innervation of the motor cortex[17], and a similar effect was found in the absence of the 5-HTT in knockout rats with increased serotonergic innervation of mPFC subdomains[24,30]. In addition, a disorganised cortical cytoarchitecture demonstrated by the Satb2, Cux1 and Tbr1 layers markers in mPFC from young 5-HTT$^{-/-}$ rats was found[24,30]. Interference of EFV with the 5-HT signaling pathway may explain the increase we found in serotonergic axonal length in the mPFC. Thus, the serotonergic system may well have been the major target for the effects of EFV we observed. Previous studies demonstrated that the 5-HTT$^{-/-}$ rats display a delay in motor development[55], comparable to the effects of perinatal EFV exposure in rats[17]. The knockout rats furthermore display increases in affective behaviour[56], lower levels of social interaction[57], and a decrease in memory for neutral information but an increase in memory for valenced (rewarding, aversive) information[58,59]. Whether perinatal EFV exposure causes a similar behavioural profile remains to be investigated.

The increase in 5-HT and catecholaminergic fibers in response to perinatal EFV exposure corresponds to our previous observation of increases in these fibers in the mPFC in rats lacking the 5-HTT[24]. This similarity suggests that the 5-HT system was the major target for the effects of EFV we observed. Previous studies demonstrated that these rats display a delay in motor development[55], comparable to the effects of perinatal EFV exposure in rats[17]. These rats furthermore display increases in affective behaviour[56], lower levels of social interaction[57], and a decrease in memory for neutral information but an increase in memory for valenced (rewarding, aversive) information[58,59]. Whether perinatal EFV exposure causes a similar behavioural profile remains to be investigated.

A limitation of this study is that we used a daily concentration of EFV (100 mg/kg) that produced plasma levels lower than the therapeutic range in humans[60]. However, such a dose has led to neurotoxic effects in rats[61,62] to the same extent as in humans[63], because the neurotoxic metabolite accumulates in brain tissue, giving our study a high translational impact. Further, our animals were exposed during the perinatal period and first postnatal week, and the analyses were performed in adult life, allowing compensatory changes to take place during this developmental period, such as reduction of cell death or induction of proliferation.

The high efficacy of EFV in inhibiting cross-infection between mother and embryo together with its low cost still makes EFV the first drug of choice for the treatment of HIV in pregnant women[1,2,3,4]. However, this may come at a cost. In rats, perinatal exposure to EFV caused a developmental delay and a clear loss of neurons in the motor cortex[17]. Our results also point to a significant neuronal loss and a disorganized cytoarchitecture in the mPFC. These findings are in line with preliminary data from the first study on EFV-exposed children in Botswana (Southern Africa), which demonstrated a compromised motor and cognitive development[15].

In conclusion, exposure of rats to EFV during pregnancy and the first week of postnatal life leads to a perturbed cytoarchitecture, and altered serotonergic and dopaminergic innervations of the infralimbic, prelimbic and cingulate cortices of their offspring. Given that the mPFC is responsible for cognitive functions, language, comprehension, decision making, planning, memory and attention, these findings may have implications for the social and cognitive functioning of adult offspring of expectant women exposed to EFV. This is of great importance when considering EFV as the drug of choice for treating pregnant, HIV-infected women.

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Conflict of interest

The authors declare that they have no conflict of interest.

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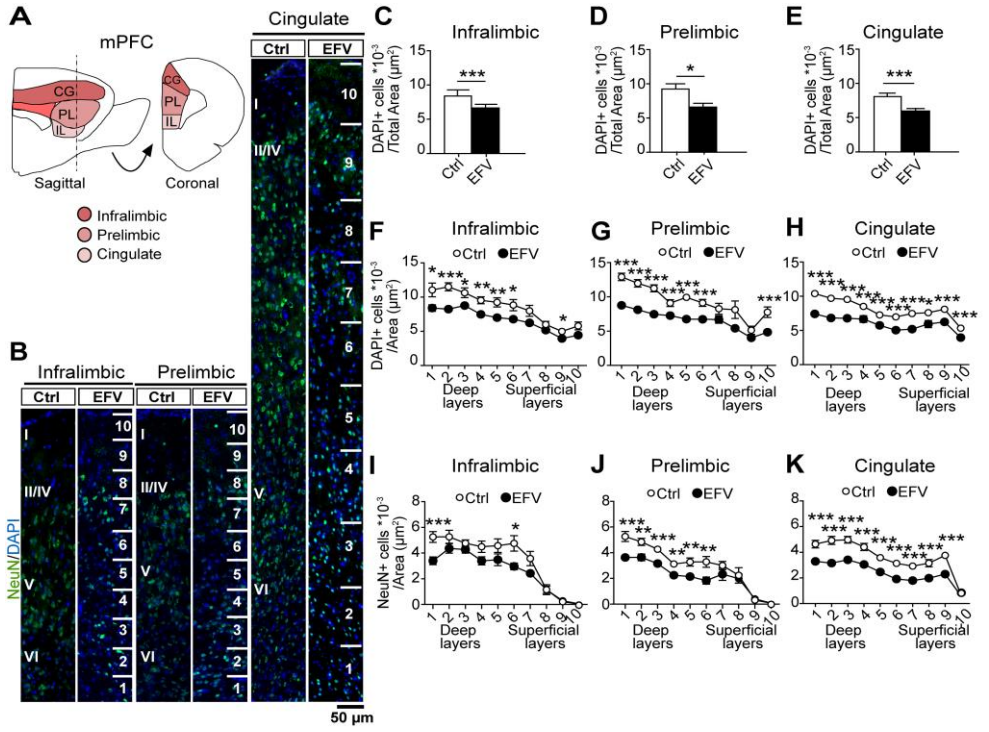


Fig. 1. Perinatal EFV exposure decreases the total number of cells and mature neurons in mPFC subareas. **(A)** Schematic of a sagittal and coronal section of the forebrain with the mPFC. **(B)** Immunostaining marking mature neurons (NeuN, green) counterstained with DAPI (blue) in coronal slices in infralimbic, prelimbic and cingulate subareas of the mPFC in EFV-exposed (n = 5) and control (Ctrl, n = 5) rats. Quantification of the number of $DAPI^+$ cells per total area in **(C-E)** mPFC subareas over the superficial and deep cortical layers **(F-H)** of the EFV–exposed group when compared with controls. **(I-K)** Quantification of mature neurons ($NeuN^+$) showing a significant decrease in $NeuN^+$ cells in deep and superficial layers in EFV–exposed and control group in the infralimbic, prelimbic and cingulate cortex. Schema adapted from McKlveen et al[64]. Data show mean (± SEM) number of cells, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (α = 5%). EFV: efavirenz; Ctrl: vehicle.

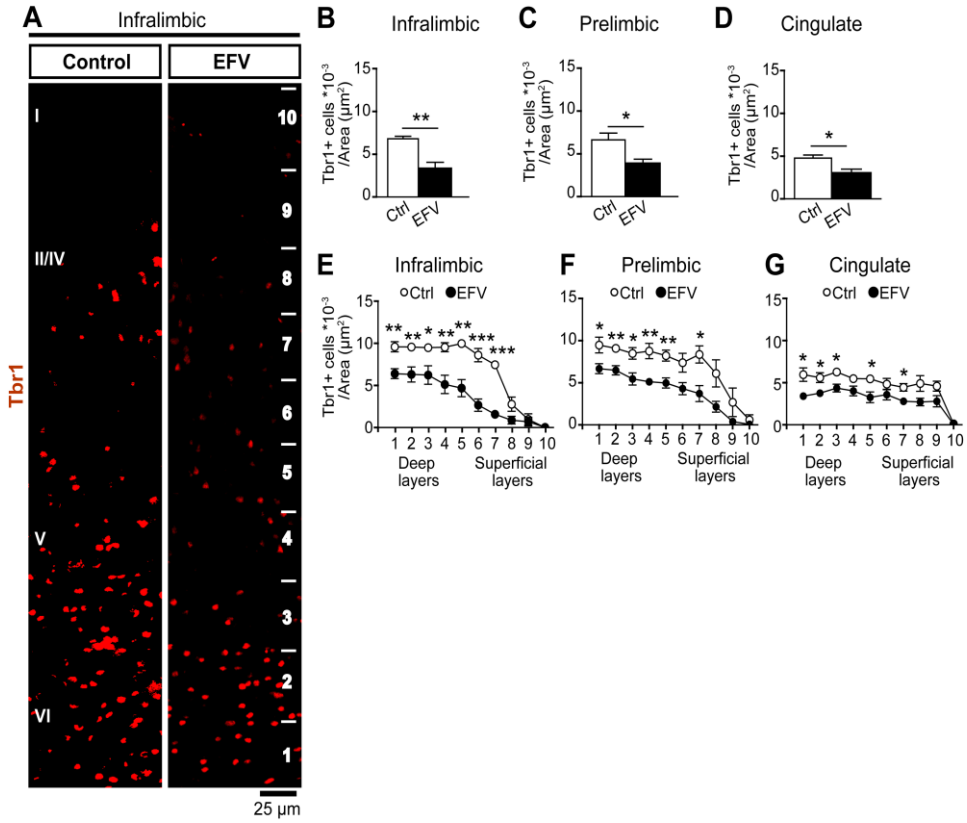


Fig. 2. Perinatal EFV exposure affects the expression of deep layer marker Tbr1 in mPFC subareas. **(A)** Immunostaining for the deep layer marker Tbr1 (red) in the prelimbic subarea of mPFC of EFV-exposed (n = 5) and control (n = 5) rats. **(B-D)** Significant decrease of $Tbr1^+$ cells in all subdomains of the mPFC cortex of the EFV–exposed group compared to controls. **(E-G)** Significant decrease in the number of $Tbr1^+$ cells in cortical deep layers of the infra-, pre- and cingulate cortex of EFV-exposed versus control rats. Data show mean (± SEM) number of cells, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA ($\alpha = 5\%$). EFV: efavirenz; Ctrl: vehicle.

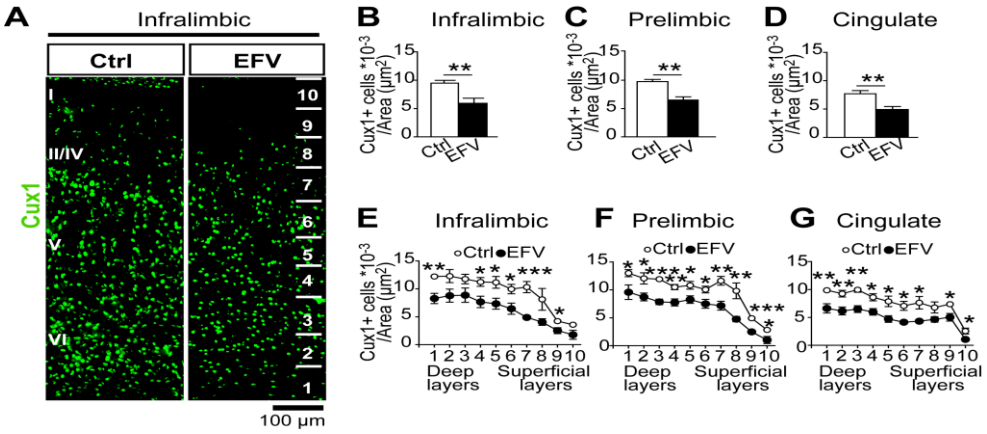


Fig. 3. Perinatal EFV exposure affects the expression of superficial layer marker Cux1 in mPFC subareas. **(A)** Immunostaining for the superficial layer marker Cux1 in infralimbic cortical subarea of the mPFC of EFV-exposed (n = 5) and control (n = 5) rats. **(B-D)** Significant decrease in number of $Cux1^+$ cells in the total area of the infralimbic, prelimbic and cingulate cortices of EFV–exposed rats compared to controls. **(E-G)** Significant decrease in the number of $Cux1^+$ cells in both cortical superficial as well as deep layers of mPFC subdomains of the EFV-exposed rats compared to controls. Data show mean (± SEM) number of cells, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA (α = 5%). EFV: efavirenz; Ctrl: vehicle.

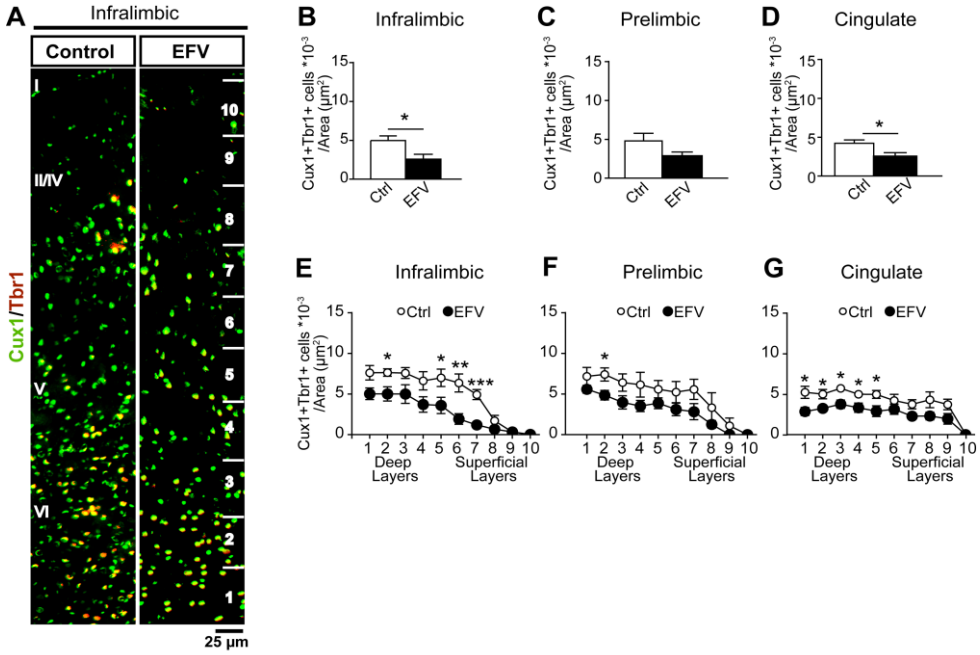


Fig. 4. Perinatal EFV exposure affects specific subsets of neurons in mPFC subareas. **(A)** Co-immunostaining for Tbr1 and Cux1 in the infralimbic subarea of mPFC of EFV-exposed (n = 5) and control (n = 5) rats. **(B-D)** Significant decrease in total number of $Tbr1^{+}/Cux1^{+}$ cells in the infralimbic and cingulate, but not prelimbic cortices in EFV-exposed *versus* control rats. Differences in the total number of $Tbr1^{+}/Cux1^{+}$ cells in the superficial and deep cortical layers of infralimbic **(E),** prelimbic **(F)** and cingulate **(G)** cortex of EFV-exposed rats compared to controls. Data show mean (± SEM) number of cells, *p < 0.05, **p < 0.01, ***p < 0.001, analyzed using One-way ANOVA.

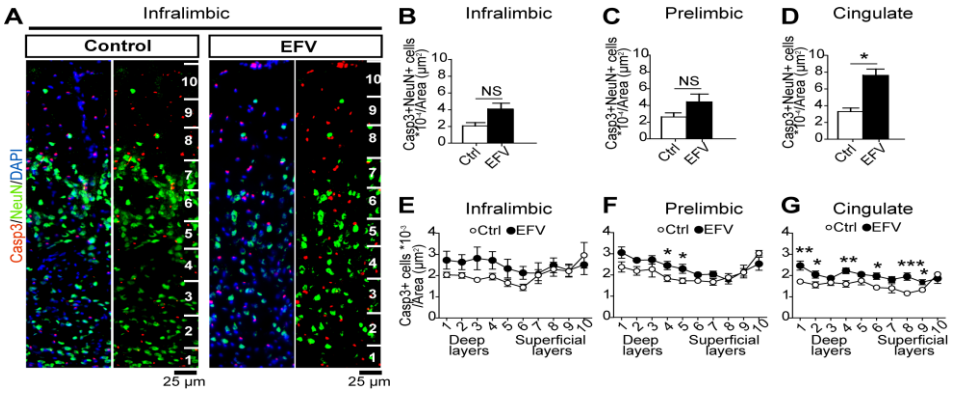


Fig. 5. Perinatal EFV exposure results in cell death in subareas of the mPFC. **(A)** Immunostaining for NeuN (green), cleaved-Caspase-3 (red) and counterstained with DAPI (blue) in the infralimbic subarea of mPFC of EFV-exposed (n = 5) and control (n = 5) rats. **(B-D)** Quantification of the number of cleaved-Caspase-3^+ neurons mPFC subdomains in EFV-exposed rats compared to controls. **(E-G)** Significant increase in cleaved-Caspase-3^+ cells per area in superficial and deep layers of the prelimbic and cingulate cortices, but not in the infralimbic cortex of EFV exposed versus control rats. Data show mean (± SEM) number of cells, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA ($\alpha = 5\%$). EFV: efavirenz; Ctrl: vehicle.

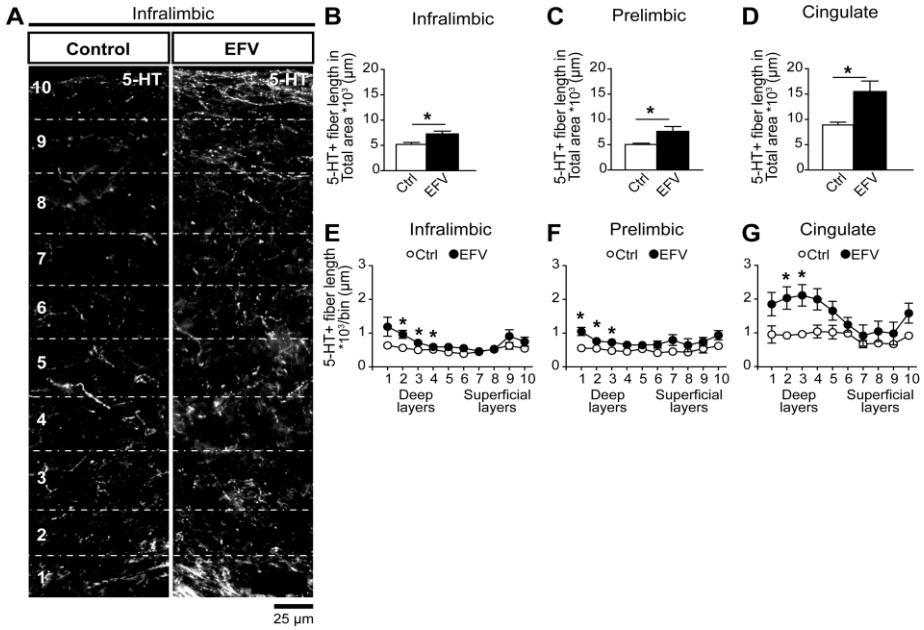


Fig. 6. Perinatal EFV exposure causes disturbances in serotonergic innervation of mPFC subareas. **(A)** Immunostaining showing 5-HT$^+$ fibers (white) in deep and superficial layers of the infralimbic cortex in EFV-exposed (n = 5) and control (n = 5) rats. **(B-D)** Quantification of serotonergic fiber length in total swatch area in all mPFC subareas in EFV-exposed animals compared to control areas. **(E-G)** A significant increase in 5-HT$^+$ fiber length in deep layers of the infralimbic, prelimbic and cingulate cortex area of EFV-exposed group compared to the controls. Data show mean (± SEM) length, *$p < 0.05$ analyzed using One-way ANOVA ($\alpha = 5\%$). EFV: efavirenz; Ctrl: vehicle; 5-HT: serotonin.

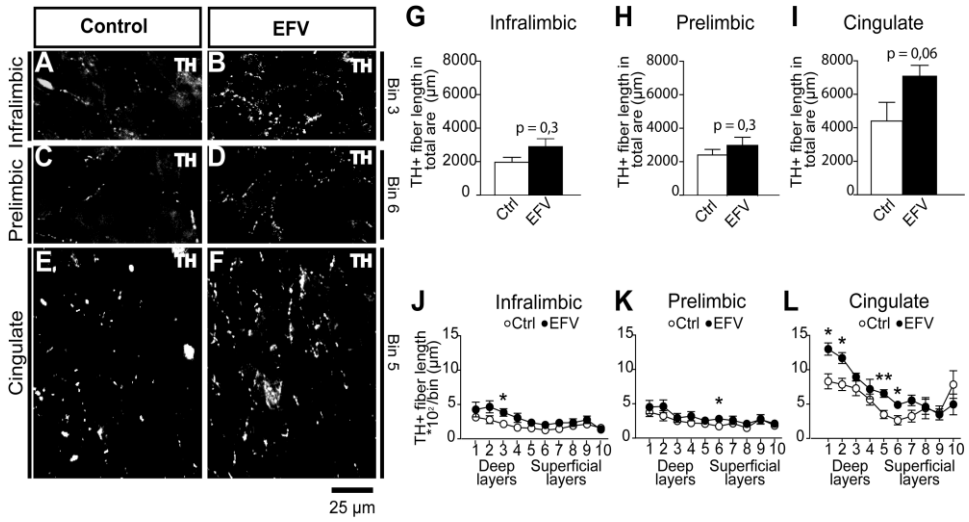
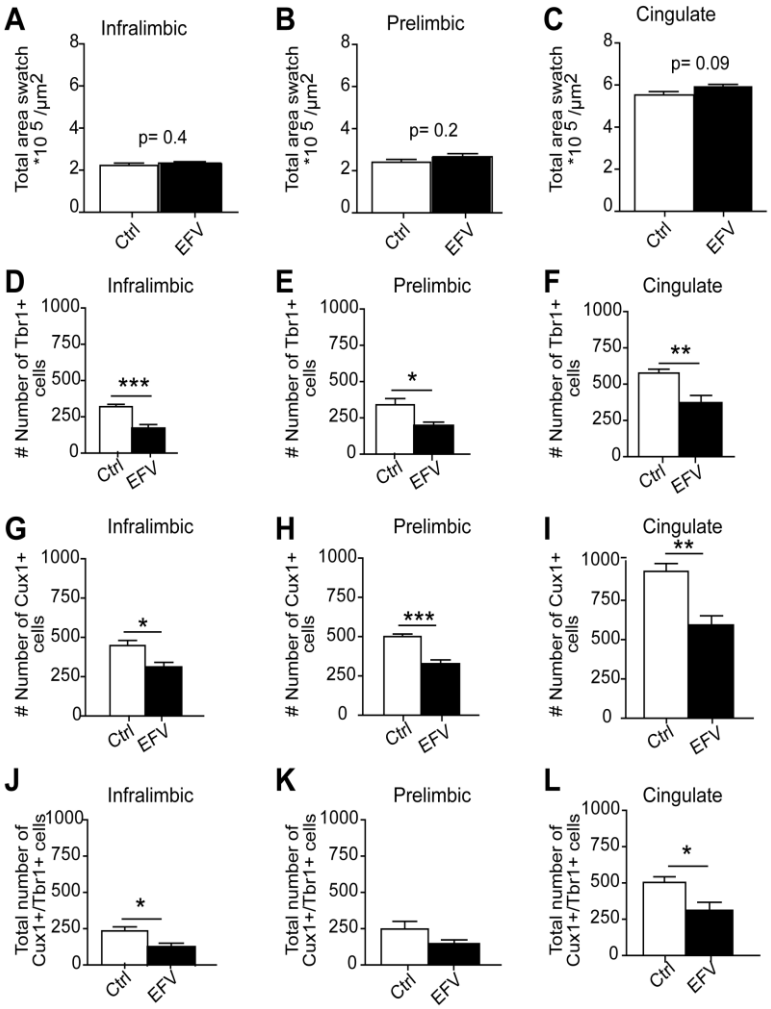


Fig. 7. Perinatal EFV exposure causes disturbances in dopaminergic innervation of mPFC subareas. **(A-F)** Immunostaining showing TH^+ fibers in infralimbic, prelimbic and cingulate cortex in EFV-exposed (n = 5) and control (n = 5) rats. **(G-I)** No significant differences in total fiber length in mPFC subareas in the EFV group compared to the control group. **(J-L)** A significant increase in TH^+ fiber length in deep layers of the infralimbic, prelimbic and cingulate cortex of EFV-exposed *versus* control rats. Data show mean (± SEM) length, *$p < 0.05$ analyzed using One-way ANOVA ($\alpha = 5\%$). EFV: efavirenz; Ctrl: vehicle; TH: tyrosine hydroxylase.



Supplemental Fig. 1. Quantification of cortical thickness and reduction in cortical layer markers expression in mPFC subareas. **(A, C)** Quantification of cortical thickness of mPFC subareas. **(D, F)** Quantification of total number of $Tbr1^+$ cells in mPFC subareas in EFV–exposed group compared to controls. **(G, I)** Quantification of total number of $Cux1^+$ cells in mPFC subareas in EFV–exposed group compared to controls. **(J, L)** Quantification of total number of $Tbr1^+/Cux1^+$ cells showing a significant decrease in $Tbr1^+/Cux1^+$ cells in the infralimbic and cingulate but not prelimbic cortex in EFV–exposed *versus* control rats. Data show mean (± SEM) number of cells, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, analyzed using One-way ANOVA ($\alpha = 5\%$). EFV: efavirenz; Ctrl: vehicle.

7

General Discussion

The dynamic course of brain development involves vital processes and adaptations that begin during the embryonic period and last a lifetime. The prenatal and early-postnatal stages of development appear to be the most vulnerable stages for aberrations[1-5]. The development of the cerebral cortex, including the evolutionarily more recent prefrontal part, starts during the embryonic period via a highly organized sequence of events, including cell proliferation, migration, differentiation, and formation of connections[5,6,7]. After birth, the cortex continues to develop and specializes into increasingly complex functions based on a precise genetic program with modulations influenced by the environment[8,9,10]. Furthermore, these events can be fine-tuned by neurotrophic factors such as serotonin (5-HT)[5,6]. 5-HT is a regulator of corticogenesis during brain development and as such a potent regulator of behavior[8].

In this thesis, prefrontal neurodevelopment was studied using wild-type animals as well as genetically modified or pharmacologically treated rodents, with a focus on the serotonergic system during embryonic development and around birth. In Chapter 2, mice were used to describe in detail the development of the medial prefrontal cortex (mPFC) at different developmental time points, in addition to comparing its development to that of the more dorsal somatosensory cortex. Once we understood the default mechanism of prefrontal development and its relationship to the serotonergic system, we used the serotonin transporter knockout (5-HTT$^{-/-}$) rat in Chapter 3 to study the role of the 5-HTT and the effect of the resulting increased 5-HT levels on the development of the mPFC at the cellular level. Additionally, we examined the interaction between the catecholaminergic and serotonergic systems during mPFC development. Chapter 4 describes increased anxiety in adolescent compared to juvenile 5-HTT$^{-/-}$ rats, which was associated with an increased 5-HT innervation of the mPFC and cell loss in deep as well as superficial layers, including mature neurons. Chapters 5 and 6 tested the effect of perinatal exposure of pregnant rats to the antiviral drug efavirenz on behavior and cortical morphology. We found a delay in motor development, reduced body weight and increased neuronal cell death in the M1 motor cortex and all subdomains of the mPFC. In this last chapter, the findings reported in chapters 2-6 and their main conclusions will be discussed and put into a broader perspective.

The role of 5-HT during cortical development

The timing of cortex development depends on the function exerted[11]. The areas responsible for higher cognitive functions develop later relative to the areas of primary functions such as visual, sensory and motor cortices[11,12]. Because of its superior and integrative functioning, the mPFC is the last area to evolve, and its development has recently been documented by magnetic resonance imaging[12]. In mice, mPFC development seems to be delayed relative to the development of more dorsal regions such as the somatosensory cortex (S1). This delay in maturation may in part be due to its serotonergic innervation. Indeed, serotonergic axons travel from the dorsal raphe nucleus in the brainstem to the mPFC at the same time as corticogenesis begins in this frontal area[4,5,6,7] (Chapters 2 and 3). Once serotonergic axons reach their frontal targets the serotonergic contribution increases, leading to a higher cellular innervation. It is important to note that the serotonergic fibers first arrive in the infralimbic areas, followed by prelimbic and cingulate cortex[8]. The compartmentalization and stratification of these mPFC subdomains occur differently when compared to the S1, with the cingulate cortex being the last area to be reached by serotonergic fibers (Chapter 2). This may explain the behavioral changes in adulthood when the connections of the cingulate cortex with other areas of the limbic system develop and these connections are associated with the control of emotions and behavior[13,14,15]. Once developed, the prelimbic and infralimbic cortices send projections back to the raphe nucleus[16,17], and create a feedback system[18,19].

The 5-HTT is expressed in cortical areas even before 5-HT projections arrive[20]. This suggesting that there must be an alternate source of 5-HT during early development. Indeed, maternal-derived 5-HT from the placenta is already present at early stages of corticogenesis[21]. Therefore, a correct cortical maturation, and appropriate 5-HT levels and innervation are necessary for the proper functioning of this circuit.

Damages caused by increased 5-HT levels during mPFC development

Changes in 5-HT levels are the basis of neuropsychiatric disorders such as schizophrenia, bipolar disorder, depression, anxiety and autism. 5-HT should be kept at appropriate levels because it is an important regulator of cortical development and emotional control[22,23]. As mentioned, one important regulator of 5-HT levels is the 5-HTT, which promotes the re-uptake of this neurotransmitter and as such regulates its extracellular

levels[23,24,25]. Alterations in the promoter of the human gene encoding 5-HTT are associated with an increase of extracellular 5-HT. Carriers of the short allele (s) of this gene initially present depressive episodes, followed by an increase of anxiety[25-30]. This circumstance in human is mimicked in the 5-HTT$^{-/-}$ rat and through selective or non-selective pharmacological animal models[33]. Altered 5-HT levels during prenatal and postnatal development affect cortical development because during this period the cortex is still at its peak of development and an excess of 5-HT leads to specific changes in mPFC gene expression[31,32,33] (Chapters 3-6). In the knockout animals, the absence of the transporter causes an increase in extracellular 5-HT[31,32], and this increase causes aberrations in the development of the serotonergic system with a lower cell density in the raphe nucleus, the origin of the serotonergic system (Chapter 3). This may result in a compensation mechanism, namely an increase of axonal projections of this system towards frontal regions (Chapter 3). At the level of the mPFC, excess of 5-HT during development caused a decrease in the number of CR cells. Furthermore, the organization of the layers was disturbed with a delay in the expression of the subcerebral projection neuron marker Satb2, i.e. a decrease at early postnatal days (Chapter 3) and an increase in early adolescence (Chapter 4). In addition, neuronal maturation was affected, and neuronal loss was provoked through activation of apoptosis (Chapters 3 and 4). This neuronal loss and a delay in cell specification, associated with an increase in serotonergic innervation in the cingulate cortex, may explain the increased anxiety identified during early adulthood of the 5-HTT$^{-/-}$ rats (Chapter 4).

Anxiety is a physiological defense mechanism and is associated with the activation of different brain areas, which mobilize the organism for an anticipatory and aversive reaction[34]. When a certain level of stress is exceeded, anxiety ceases to be adaptive and becomes pathological[35,36,37]. The mPFC plays an important role in the coordination of behavioral and physiological stress responses[38,39], especially the cingulate cortex, most likely through its reciprocal connections with the amygdala and other parts of the limbic system[17,40,41,42]. In systems with an affected 5-HTT these structures are also disturbed and characterized by cell loss (Chapter 3)[45]. Interestingly, anxiety emerges only between PND25 and PND35 in 5-HTT$^{-/-}$ rats and not earlier (Chapter 4). This may be due to the relatively late maturation of the mPFC. Apparently, connections between the mPFC and limbic areas seem to depend on proper mPFC maturation which begins during the embryonic period and lasts until adulthood[43,44,45] (Chapters 2 and 4).

Emerging compensatory mechanisms, such as dynamic modulation of the expression of 5-HT receptors in different cortical areas, can be key to the eventual effects of excess 5-HT[46]. These receptors may stimulate cell proliferation or activate apoptotic pathways[47]. The serotonergic receptors 5-HT_{1A}, $5HT_{2A}$, 5-HT_{2C} and 5-HT_{3A} have been associated with depressive behavior and increased anxiety when 5-HT levels are increased [46,47]. For example, the selective pharmacological block of the transporter during the embryonic period, via fluoxetine, increases expression of the 5-HT_{3A} receptor, which has been associated with the occurrence of autism[48,49]. Studies have focused on avoiding these compensatory effects of the blockade or absence of 5-HTT. Specific blockers of 5-$HT_{2A/C}$ receptors administered at early-postnatal periods were able to prevent the pharmacologically induced alterations in cortical areas, and as such inhibited depression and anxious behavior in adult life[50].

The change in 5-HT levels not only caused alterations in cortical development and in the serotonergic system itself, but also interfered with the development of another system, the dopaminergic system (Chapters 3, 5 and 6). Following the increase in 5-HT levels, the loss of cells in the ventral tegmental area (VTA) and in the substantia nigra compact (SNc) promoted an increase of the dopaminergic axon projections to the mPFC, possibly as a compensatory mechanism (Chapter 3). Since there are contacts between dopaminergic fibers and CR cells, the increased dopaminergic mPFC innervation may lead to even more disturbances in cortical development (Chapter 3). This is interesting because also during corticogenesis dopamine (DA) participates in several developmental processes[51,52,53]. Such an interaction between systems has previously been demonstrated only through pharmacological blocks of dopaminergic, noradrenergic or serotonergic levels, receptors and transporters[54,55]. The addition of methylphenidate, an inhibitor of DA and noradrenaline transporters, together with the 5-HTT inhibitor citalopram led to a decrease in extracellular 5-HT levels and increased levels of DA in the PFC of rats[55]. The integration of different systems, as exemplified by a loss of cells in the dopaminergic system in the absence of 5-HTT (Chapters 3 and 4), should be carefully monitored when perinatally administering drugs (Chapters 5 and 6). The interaction of various transmitter systems during development could cause even more cortical damage than initially thought and can last into adulthood (Chapters 5 and 6).

Effects of pharmacological agents on cortical development

In many cases the use of medication during pregnancy cannot be avoided, for example when trying to prevent cross-infection between mother and child by HIV during the pre- and perinatal periods. The drug most commonly used in this respect, efavirenz (EFV)[57,58], is a non-nucleoside reverse transcriptase inhibitor that blocks HIV replication. Additionally, EFV interacts with the serotonergic system through not only the 5-HTT but also via stimulation or inhibition of various 5-HT receptors[59,60]. These pharmacological targets can be the basis of the side effects presented by patients (or their offspring) and found in animal models, such as increased levels of anxiety, depression, hallucinations and aggressiveness[61-66].

Previous studies on possible damage caused by EFV in the immature brains of humans and nonhuman primates have given inconclusive results[67]. *In vitro* studies have demonstrated a clear effect of EFV on the death of progenitor cells from different brain areas, and *in vivo* EFV-induced brain toxicity has been found in adult animals[68-72]. We exposed animals to EFV throughout the prenatal and lactation periods and evaluated the side effects of the drug on the adult brain. Behavioral tests during early postnatal ages, adolescence and adulthood showed a delay in motor and reflex development (Chapter 5). A similar outcome was obtained in a large-scale study on African children exposed to EFV. These children had a worse motor development when compared to children not exposed to EFV[73].

At the cellular level, EFV intake during prenatal and early postnatal periods elicited cellular apoptosis in all cortical layers of the mice. In the adult primary motor cortex (Chapter 5) and mPFC (Chapter 6), these cells were mature neurons or astrocytes, which also regulate neuronal survival through the secretion of neurotrophic factors[69,72]. At the same time, the excess of serotonergic innervation, associated with EFV cell toxicity, caused disturbances in the cortical organization and promoted an increase in dopaminergic innervation, which was evaluated in the M1 (Chapter 5) and mPFC (Chapter 6).

Interestingly, the observed behavioral effects of EFV resemble those previously found in response to perinatal exposure of rats to selective serotonin reuptake inhibitors (SSRIs) and in 5-HTT$^{-/-}$ rats[33], albeit milder. This, in combination with the increased serotonergic innervation of the mPFC as also observed in the 5-HTT$^{-/-}$ rats (Chapter 3), implies that the effects of EFV are mainly mediated through the serotonergic system. The milder behavioral consequences of perinatal EFV exposure compared to perinatal

SSRI exposure may relate to the fact that SSRIs increase 5-HT levels that can bind to all 15 5-HT receptors, whereas EFV more specifically targets the postsynaptic 5-HT_{2A} receptor[59,60]. In support, a 5-HT_{2A} receptor antagonist treatment was found to partially block the effects of perinatal SSRI exposure on behavior in offspring[50].

These data demonstrate an important role of 5-HT in cortical development and control of behavior, and especially point to the danger such drugs can pose during brain development. Such a risk factor should be considered when prescribing or using these types of medication perinatally.

In conclusion, events occurring during early stages of cortical development determine the basic neurobiological architecture and function of the brain, and eventually define behavior in adult life. For proper brain functioning, these events require adequate levels of 5-HT. Identifying when and where developmental events occur, offers a broadening of our understanding of brain development and highlights the mechanisms underlying neurodevelopmental disorders. The stimuli transmitted to the brain during the pre- and postnatal periods, and also during the other stages of life, affect the functioning of neurons and neural circuits. In addition, the mechanisms of brain plasticity induced by environmental stimuli during the pre- and postnatal periods add to the understanding of how the cortex develops and how prenatal events are reflected in adult behavior. The ultimate goal of this thesis was to identify some of these neurodevelopmental mechanisms in the maturation of the mPFC, and to study the effects of the serotonergic system on these mechanisms and how disturbances in this system are translated into various diseases in which the PFC plays a major role. As such, we hope to have contributed to the understanding of how the structure of the PFC develops and how these developmental events are subject to various risk factors like increased 5-HT levels, either via genetic or pharmacological influence, causing structural damage that will persist throughout adult life and become apparent as perturbed behavior. To provide a graphical overview, the results of the studies reported in this thesis are summarized in Fig. 1.

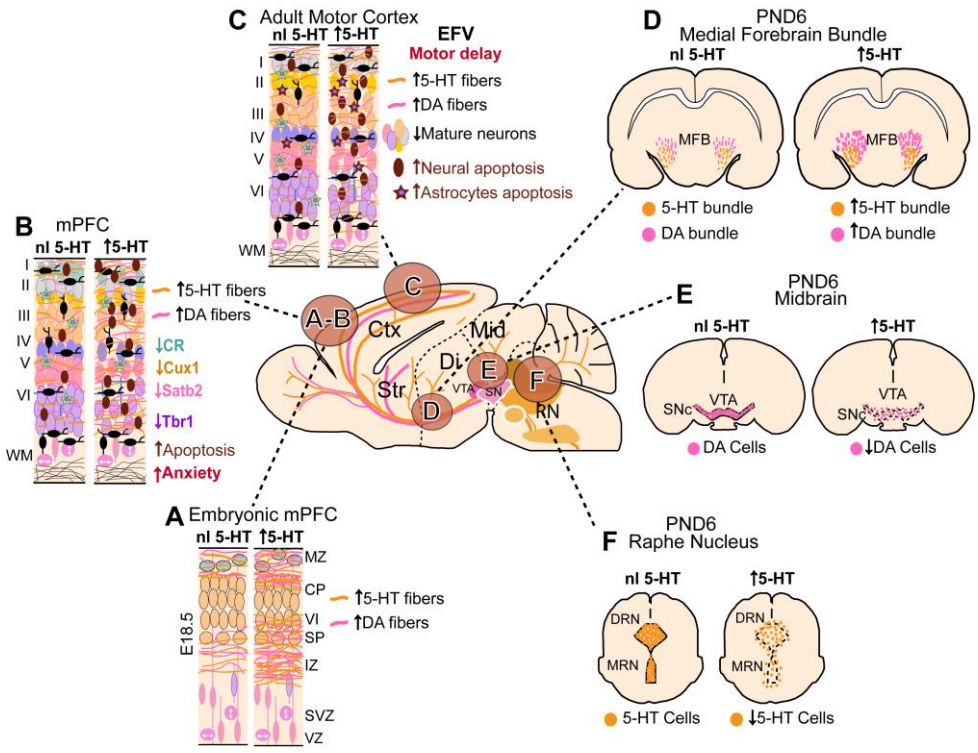


Fig. 1. Effects of 5-HT increase on brain development. **(A)** - The increase of extracellular 5-HT causes an increase in serotonergic and dopaminergic innervation in cortical areas especially the MZ, where they contact with CR cells in the embryonic mPFC. (**B) -** Already in the postnatal mPFC, the increase of 5-HT induces a neuronal cell death program, and altered organization of the cortical layers, demonstrated by decreasing levels of the transcription factor layer markers Cux1 (superficial), Satb2 (superficial and deep) and Tbr1 (deep). The increase of serotonergic and dopaminergic innervation remains. These changes lead to an increase in anxious behavior in adulthood. (**C) -** During the development of the motor cortex, the increase in 5-HT levels, via EFV, caused an increase in dopaminergic innervation, and neuronal and astrocyte death, which was reflected by a delay in motor and reflex development. (**D) -** The increase in 5-HT levels in the medial forebrain bundle was established by the increase in the number and thickness of serotonergic and dopaminergic fascicles in this region early postnatally (P6). (**E) -** The increase in 5-HT levels during embryonic development caused a decrease in the number of dopaminergic neurons in the midbrain, which were identified at P6. (**F) -** Finally, the increase in 5-HT levels during the embryonic period causes a significant decrease in cell density in the dorsal and medial raphe nuclei, being the origin of the serotonergic system at P6. 5-HT, serotonin; nl 5-HT, normal levels of serotonin; DA, dopamine; MZ, marginal zone; CP, cortical plate; SP, subplate; IZ,

intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter; CR, Cajal-Retzius cells; RN, rape nucleus; SN, substantia nigra; VTA, ventral tegmental area; DRN, dorsal raphe nucleus; MRN, medial raphe nucleus; Str, striatum; Mid, midbrain; Di, diencephalon, Ctx, cortex.

Future prospects

The early years of human development establish the basic neurobiological architecture and function of the brain. This initial period of development, from conception to the first years of age, is crucial as it defines physical and mental health in later life. The stimuli transmitted to the brain by sensory circuits during pre- and postnatal periods, and also in later stages of life, shape the function of neurons and neural circuits. For instance, altered levels of 5-HT are a risk factor for disorders such as depression and anxiety. Currently, the treatment of these disorders involves only a standardized treatment with mostly symptom management, rather than taking the cause and/or other transmitter systems into consideration. Timely preventing cortical aberrations, e.g. caused by deviations in 5-HT levels, may provide a more attractive therapy. Therefore, knowledge of the biological framework that underpins these disorders is essential. Longitudinal studies that describe all aspects of (human) brain development are crucial for the understanding of the defects that occur in neurological disease, but maybe more so in neurodevelopmental disorders. The exact timing of the occurrence of deficits and the establishment of the precise critical developmental window in which these deficits start to manifest themselves is vital for determining the best treatment strategy. In this thesis, we focused on the role of 5-HT in cortical development and the effects of alterations in 5-HT levels during development on behavior. However, as described in Chapter 3, during development 5-HT interacts with the catecholaminergic system and most likely also with other systems. We therefore have to be careful with choosing perinatal treatment regimens since pharmacologic agents are likely to perturb multiple systems and may thereby alter fundamental biological processes at a systems level. Although animal models have their limitations, they allow us to test these hypotheses and have high translational value. Future studies on human as well as animal models will hopefully shed more light on the mechanisms underlying PFC development in health and disease, and more specifically on the role of 5-HT and other transmitters in these processes. By performing basic research in animal models with high translational value, we hope to have contributed to the understanding of the (serotonergic) mechanisms underlying

PFC development that when disturbed are known to play an important role in the etiology of many brain disorders.

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Appendix

Summary

The prefrontal cortex is the area of the brain responsible for the complex control of higher cognitive functions. Correct cortical development depends on a series of orchestrated events that begin in the embryonic period, linger during the adolescent phase and result in an intricate six-layered structure in adulthood. The sequence of events occurring during cortical development covers cell proliferation, migration, differentiation, formation of various connections and synaptic pruning. All of these events are timely controlled by intrinsic and extrinsic factors, including serotonergic neuromodulation. Serotonin is an important neurotrophic factor during development that has its origin in the brainstem and serotonergic neurons project to diverse regions in the brain, among them the prefrontal cortex. Serotonin participates in the regulation of neurodevelopmental processes such as cell proliferation and maturation, and programmed cell death. Therefore, appropriate levels of serotonin should be maintained for correct cortical development and disturbances in serotonin levels are associated with a number of disorders.

Chapter 2 presents the cortical events occurring during normal development of the mouse brain and compares the development of various cortical areas. Events such as cell proliferation and the appearance of the cortical layers appear to coincide with the development of the serotonergic and dopaminergic systems and their arrival in prefrontal and somatosensory areas. However, frontal development was delayed when compared to the more dorsally located somatosensory cortex. This shows that the prefrontal cortex needs a longer time to develop than the somatosensory cortex, perhaps due to the large number of connections that the prefrontal cortex has to establish with other brain areas.

After examining how events normally occur during prefrontal cortical development, we next studied how increased cortical serotonin levels would affect normal development. In **Chapter 3**, we used rats lacking the serotonin transporter (5-HTT$^{-/-}$ rats), and found that these rats exhibited an increase in serotonergic innervation of the prefrontal cortex and changes in prefrontal cortical differentiation. Furthermore, we observed that the number of cells was decreased in the dorsal raphe nucleus in the brainstem, the origin of the serotonergic system. We further demonstrated an interrelationship between the serotonergic system and the dopaminergic system, since an increase in

also dopaminergic innervation was found in the prefrontal areas of the 5-HTT$^{-/-}$ rats, in addition to a loss of cells in the origins of the dopaminergic system, the ventral tegmental area and substantia nigra. The cortical changes induced by elevated levels of serotonin were further explored in **Chapter 4**. Excessive serotonin levels were found to be associated with altered cellular migration and neuronal maturation, and active apoptotic pathways. These developmental changes caused a disorganized cortical structure at early postnatal age, which remained during adolescence. This was paralleled by an increase in anxious behavior during adolescence that persisted into adulthood. Together, the results demonstrate that serotonin plays a key role in embryonic cortical development and the need to maintain adequate levels of this neuromodulator.

Chapters 5 and **6** show how perinatal serotonergic drug exposure can alter brain development in the offspring. We tested the medication most commonly used to treat HIV-positive pregnant women, efavirenz (EFV). EFV blocks viral replication and decreases the chance of cross-infection between mother and child during the pre- and perinatal period to 5%. EFV acts as an agonist or antagonist of several receptors and transporters, including serotonergic ones. This medication was administered to pregnant rats throughout the embryonic and lactation periods, and the offspring was studied across a number of developmental stages. EFV exposure was associated with a delay in motor and reflex development, and increased serotonergic innervation as well as cell death in the motor cortex. Similar findings were obtained in the prefrontal cortex, namely an increase in serotonergic and dopaminergic innervation, and a marked loss of neurons. Additionally, the cytoarchitectural organization of the prefrontal cortex was altered. Together, these chapters demonstrate that EFV changes the development of the immature cortex, and that these changes remain until adulthood and are reflected by a delay in reflex and motor development. Further studies are needed to fully elucidate the cellular and behavioral side effects caused by EFV toxicity in the immature brain. In the meantime, caution is required when prescribing this type of medication for pregnant women.

The main purpose of this thesis was to demonstrate the importance of serotonin for embryonic cortical development, and in what sense corticogenesis is vulnerable to genetically- or pharmacologically-induced alterations in the levels of this neurotrophic factor. Excess serotonin may

cause damage to the formation and correct organization of the cortex, and this is reflected by altered (aggressive, depressed, anxious) behavior in adulthood. With our studies we hope to have contributed to the understanding of the development of the prefrontal cortex and highlighted the care that should be taken when prescribing or using drugs during cortical development, especially in the gestational period.

Samenvatting

De prefrontale cortex is het gebied van de hersenen dat verantwoordelijk is voor de complexe controle van hogere cognitieve functies. Een juiste corticale ontwikkeling is afhankelijk van een reeks samenhangende gebeurtenissen die beginnen in de embryonale periode, verder veranderen tijdens de adolescente fase en resulteren in een ingewikkelde, zes-laagse structuur op volwassen leeftijd. De volgorde van de gebeurtenissen die zich voordoen tijdens de corticale ontwikkeling omvatten achtereenvolgens celproliferatie, -migratie, -differentiatie en -rijping, en vorming en snoeien van synaptische verbindingen. Al deze gebeurtenissen worden op de juiste tijd gereguleerd door intrinsieke en extrinsieke factoren, waaronder serotonerge neuromodulatie. Serotonine is een belangrijke neurotrofische factor in ontwikkeling die zijn oorsprong in de hersenstam heeft en van waaruit projecties naar verschillende regio's in de hersenen gaan, waaronder de prefrontale cortex. Serotonine neemt deel aan de regulatie van neuro-ontwikkelingsprocessen zoals celproliferatie en -rijping, en geprogrammeerde celdood. De juiste niveaus van serotonine zijn belangrijk voor een correcte corticale ontwikkeling en verstoringen in serotonineniveaus zijn geassocieerd met een aantal aandoeningen.

Hoofdstuk 2 laat zien hoe corticale gebeurtenissen optreden tijdens de normale ontwikkeling van de hersenen van de muis en vergelijkt de ontwikkeling van verschillende corticale gebieden. Gebeurtenissen zoals celproliferatie en het verschijnen van de corticale lagen lijken samen te vallen met de ontwikkeling van de serotonerge en dopaminerge systemen en hun aankomst in prefrontale en somatosensorische gebieden. Echter, de ontwikkeling van de prefrontale cortex was vertraagd ten opzichte van de meer dorsaal gelegen somatosensorische cortex. Dit geeft aan dat de prefrontale cortex een langere tijd nodig heeft om zich te ontwikkelen dan de somatosensorische cortex, misschien vanwege het grote aantal verbindingen die de prefrontale cortex moet maken met andere hersengebieden.

Na het onderzoek naar de processem die optreden tijdens de normale ontwikkeling van de prefrontale cortex, hebben we vervolgens het effect van verhoogde corticale serotonineniveaus op dit proces bestudeerd. In **Hoofdstuk 3** hebben we ratten zonder de serotonine transporter (5-HTT$^{-/-}$ ratten) gebruikt, welke worden gekenmerkt door hoge extracellulaire serotonineniveaus in de hersenen. We vonden dat deze ratten een toename

van serotonerge innervatie van de prefrontale cortex en veranderingen in prefrontale corticale differentiatie vertoonden. Verder stelden we vast dat het aantal cellen was afgenomen in de dorsale raphekern in de hersenstam, waar de serotonerge neuronen zijn gelokaliseerd. Tevens toonden we een verband aan tussen het serotonerge systeem en het dopaminerge systeem, aangezien er ook een toename van dopaminerge innervatie werd gevonden in de prefrontale gebieden van de 5-HTT$^{-/-}$ ratten, naast een verlies van cellen in de gebieden waar het dopaminerge systeem is gelokaliseerd, namelijk het ventrale tegmentale gebied en de substantia nigra. De corticale veranderingen veroorzaakt door de verhoogde niveaus van serotonine werden verder onderzocht in **Hoofdstuk 4**. We vonden dat de overmaat aan serotonine de celmigratie, neuronale rijping en actieve apoptotische paden veranderden, wat vervolgens leidde tot een ongeorganiseerde corticale structuur vanaf jonge leeftijd tot aan volwassenheid. Dit ging gepaard met een toename van angstig gedrag vanaf adolescente leeftijd tot aan volwassenheid. Deze resultaten tonen aan dat serotonine een belangrijke rol speelt tijdens de embryonale neuronale ontwikkeling en geven de noodzaak aan om een adequaat serotonineniveau te handhaven .

De **Hoofdstukken 5** en **6** laten zien hoe perinatale blootstelling aan serotonerge geneesmiddelen de hersenontwikkeling bij de nakomelingen kan veranderen. We hebben het medicijn getest dat het meest wordt gebruikt om HIV-positieve, zwangere vrouwen te behandelen, te weten efavirenz (EFV). EFV blokkeert virale replicatie en vermindert de kans op kruisbesmetting tussen moeder en kind tijdens de pre- en perinatale periode tot 5%. EFV werkt als een agonist of antagonist van verschillende receptoren en transporteurs, waaronder serotonerge. Deze medicatie werd toegediend aan zwangere ratten gedurende de embryonale- en lactatieperiode, en de nakomelingen werden in een aantal ontwikkelingsstadia bestudeerd. Blootstelling aan EFV ging gepaard met een vertraging in de ontwikkeling van reflexen en motorgedrag, en verhoogde serotonerge innervatie en celdood in de motorische cortex. Vergelijkbare bevindingen werden verkregen in de prefrontale cortex, namelijk een toename van serotonerge en dopaminerge innervatie, en een duidelijk verlies van neuronen. Bovendien was de cytoarchitectonische organisatie van de prefrontale cortex veranderd. Tezamen tonen deze hoofdstukken aan dat EFV de ontwikkeling van de onvolwassen cortex verandert, en dat deze veranderingen aanhouden tot aan volwassen leeftijd en gepaard gaan met een vertraging in reflex- en motorische ontwikkeling. Verdere studies zijn nodig om de cellulaire en gedragsmatige bijwerkingen

veroorzaakt door vroege blootstelling aan EFV op te helderen. Tot die tijd is voorzichtigheid geboden bij het voorschrijven van dit soort medicatie voor zwangere vrouwen.

Het belangrijkste doel van dit proefschrift was om het belang van serotonine voor embryonale corticale ontwikkeling aan te tonen, en in welke zin corticogenese kwetsbaar is voor genetisch- of farmacologisch-geïnduceerde veranderingen in de niveaus van deze neurotrofe factor. Overtollige serotonine kan de vorming en de juiste organisatie van de cortex beïnvloeden, en dit wordt op volwassen leeftijd weerspiegeld in veranderd (agressief, depressief, angstig) gedrag. Met onze studies hopen we een bijdrage te hebben geleverd aan het begrip van de ontwikkeling van de prefrontale cortex en hebben we de aandacht gevestigd op de zorg die moet worden besteed aan het voorschrijven of het gebruik van geneesmiddelen tijdens de corticale ontwikkeling, met name in de zwangerschapsperiode.

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LIDIANE GARCIA

ABOUT ME

In 2009, Lidiane Pereira Garcia, through a four-year PROUNI grant, completed a Bachelor's degree in Physical Therapy at the University of Uberaba. During graduation, she worked for two years as a tutor in the Department of Anatomy and Neuroanatomy and was part of the Academic League of Orofacial Pain. She was the founding president of the Sorria Group and provided free hospital and clinic care to the population served by the Unified Health System (SUS) at the Medical Institute of Orthopedics and Traumatology of Ituiutaba (IMOT), Nursing Home Pedro and Paulo, UNIUBE University Hospital and Dr. Helio Angotti Cancer Hospital. She is specialist in Cardiac and Pulmonary Rehabilitation in Neonates and Pediatrics at the Intensive Care Unit at Redentor College and the Federal University of Triangulo Mineiro. She is also a specialist in Permanent Education in Health at the Oswaldo Cruz Foundation (FioCruz). In 2010, she received a two-year CAPES/REUNI-28201084 grant to complete her Master's degree in the Department of Physiology and Neurophysiology of the Federal University of Triangulo Mineiro, with a background in Cell Membrane Physiology, drugs tests, Pharmacology, and Biochemistry. Then in 2014, started her PhD at the Department of Molecular Animal Physiology at Radboud University after receiving a four-year grant from the Science Without Borders program funded by the Brazilian government agency for the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, BEX11914 /13-0) with a background in brain neurodevelopment, where she stays until the moment.

List of Publications

Leite CF, Lopes CS, Alves AC, Fuzaro CS, Silva MV, Oliveira LF, **Garcia LP**, Farnesi TS, Cuba MB, Rocha LB, Rodrigues V Jr, Oliveira CJ, Dias da Silva VJ. Endogenous resident c-Kit cardiac stem cells increase in mice with an exercise-induced, physiologically hypertrophied heart. Stem Cell Res. 2015 Jul;15(1):151-64. doi: 10.1016/j.scr.2015.05.011.

Garcia LP[*], Witteveen JS[*], Middelman A, van Hulten JA, Martens GJM, Homberg JR, Kolk SM. Perturbed developmental serotonin signaling affects prefrontal catecholaminergic innervation and cortical integrity. Mol Neurobiol. 2019 Feb; 56(2): 1405-1420. doi: 10.1007/s12035-018-1105-x.

van de Wijer L[*], **Garcia LP**[*], Hanswijk SI, Rando J, Middelman A, Ter Heine R, Mast Q, Martens GJM, van der Ven AJAM, Kolk SM[#], Schellekens AFA[#], Homberg JR[#]. Neurodevelopmental and behavioral consequences of perinatal exposure to the HIV drug efavirenz in a rodent model. Transl Psychiatry. 2019 Feb 11; 9(1): 84. doi: 10.1038/s41398-019-0420-y.

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List of Abbreviations

5-HT	5-hydroxytryptamine;Serotonin
5-HTT	Serotonin Transporter
8-OH-EFV	8-Hydroxy-efavirenz
AADC	Aromatic Amino Acid Decarboxylase
ADHD	Attention Deficit Hyperactivity Disorder
ART	Antiretroviral Therapy
ASD	Autism Spectrum Disorder
ATP	Adenosine Triphosphate
AUC	Area-Under-the-Curves
BB	Blocking Buffer
BrdU	5-Bromo- 2-deoxyUridine
C	Contact
c-AMP	Cyclic Adenosine Monophosphate
CG	Cingulate
cl-Casp3	Cleaved-Caspase-3
CNS	Central Nervous System
CR	Cajal-Retzius
CRN	Caudal Raphe Nucleus
CSMN	Corticospinal Motor Neuron
cSN	Compact Substancia Nigra
Ctrl	Control
Ctx	Cortex
DA	Dopamine
DAPI	4′,6-diamidino-2-phenylindole
DRN	Dorsal Raphe Nucleus
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
E	Embryonic day
ED	Eating/Drinking
EFV	Efavirenz
FBS	Fetal Bovine Serum
GABA	Gamma-Aminobutyric Acid
GD	Gestational Day
GE	Ganglionic Eminence
Hind	Hindbrain
HIV	Human Immunodeficiency Virus
IL	Infralimbic
IZ	Intermediate Zone
LG	Licking/Grooming
M1	Primary Motor Cortex
MFB	Medial Forebrain Bundle
Mid	Midbrain

mPFC	Medial Prefrontal Cortex
MRN	Median Raphe Nucleus
MZ	Marginal Zone
N	Nursing
NA	Norepinephrine
NDS	Normal Donkey Serum
NeuN	Neuronal Nuclear Protein
NGS	Goat Serum
NHS	Normal Horse Serum
OCD	Obsessive-Compulsive Disorder
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde Solution
PFC	Prefrontal Cortex
PL	Prelimbic
PND	Postnatal Day
PP	Preplate
PPI	Prepulse Inhibition
PTSD	Post-Traumatic Stress Disorder
RRF	Retrorubral Field
RRN	Rostral Raphe Nucleus
S1	Primary Somatosensory
SG	Self-Grooming
SP	Subplate
SSRIs	Selective Serotonin Reuptake Inhibitors
Str	Striatum
SVZ	Subventricular Zone
Tel	Telencephalon
TH	Tyrosine Hydroxylase
VTA	Ventral Tegmental Area
VZ	Ventricular Zone
WHO	World Health Organization
WM	White Matter
WT	Wild-Type
X	Out of nest

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For a successful research institute, it is vital to train the next generation of young scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School for Cognitive Neuroscience (DGCN), which was officially recognised as a national graduate school in 2009. The Graduate School covers training at both Master's and PhD level and provides an excellent educational context fully aligned with the research programme of the Donders Institute.

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Positions outside academia spread among the following sectors:

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- specialists in a psychological environment, e.g. as specialist in neuropsychology, psychological, diagnostics or therapy,
- higher education as coordinators or lecturers.

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